DESCRIPTION

GENE AND PEPTIDE FOR TRANSCRIPTIONAL REPRESSOR

Technical Field

The present invention relates to a peptide or protein capable of converting a transcription factor into a transcriptional repressor, a gene encoding such peptide or protein, a chimeric protein in which the aforementioned peptide or protein is fused to a transcription factor, a chimeric gene in which the gene encoding a peptide or protein is fused to a gene encoding a transcription factor, a recombinant vector having such chimeric gene, and a transformant comprising such recombinant vector.

Background Art

In the past, the antisense method and the ribozyme method were known as means of repressing the transcription of organisms' genes to mRNA or repressing the expression of such genes. Research on the application of these techniques to, for example, repression of the expression of genes that can cause diseases, such as oncogenes, or improvement of plants, has made progress. In the antisense method, antisense DNA or RNA that is complementary to a specific site of a target gene, transcription of which is to be repressed, or mRNA to which the target gene has been transcribed, is employed. Existing antisense DNA or RNA, however, cannot be used for repressing the expression of genes other than the aforementioned target gene. Thus, new antisense DNA or RNA needs to be prepared in accordance with sequences of other target genes. In the case of the ribozyme method, the target DNA or mRNA cannot be cleaved with a ribozyme unless such ribozyme is designed to have a complementary sequence in order to bind to the target DNA or mRNA and to be capable of cleaving it at a predetermined position. Even when the ribozyme is designed to cleave the target gene, an excessive sequence is sometimes added to a transcribed ribozyme, which may result in the loss of ribozyme activity when, for example, it is ligated to a promoter such

as the cauliflower mosaic virus 35S promoter and a transcription terminator sequence to construct a vector for introduction, and the resultant is actually introduced to a plant cell. In these conventional techniques, identification of the target gene and determination of its nucleotide sequence have always been indispensable. A method for repressing the gene expression via the gene knock-out technique has also been available, although this technique could not be applied to, for example, amphidiploid plants.

The present inventors have found that *Arabidopsis* proteins, namely, AtERF3, AtERF4, AtERF7, and AtERF8 are transcription factors which can significantly repress transcription of genes via an approach completely different from those of the aforementioned conventional techniques. They constructed effector plasmids comprising the genes encoding the aforementioned proteins and DNAs cleaved therefrom, and they introduced the resultants to plant cells. Thus, they actually succeeded in repressing gene transcription (JP Patent Publication (Kokai) Nos. 2001-269177 A, 2001-269178 A, 2001-292776 A, and 2001-292777 A). Further, the present inventors subjected a gene encoding tobacco ethylene responsive element binding factor (ERF) 3, which is a Class II ERF genes (JP Patent Publication (Kokai) No. 2001-269176 A), a gene encoding *Oryza sativa* Os ERF3 protein (JP Patent Publication (Kokai) No. 2001-269179 A), and genes encoding *Arabidopsis thaliana* ZAT10 and ZAT11, which are Zn finger protein genes, to a test similar to the aforementioned test. As a result, they found that transcription of target gene was repressed. They demonstrated the existence of a conserved motif (L/F)DLN(L/F)(X)P (X denotes any amino acid residue) in proteins or peptides encoded by these genes, although the nucleotide sequences of these genes are different from each other (The Plant Cell, Vol. 13, 1959-1968, August, 2001).

An object of the present invention is to provide a simple and extensively applicable means for repressing transcription of genes, which eliminates the need for designing new DNA or RNA in each case in accordance with the nucleotide sequence of the target gene, unlike the conventional antisense and ribozyme methods. It is another object of the present invention to provide a peptide for facilitating the repression of

transcription of genes and a gene thereof by further advancing the research concerning the transcriptional regulatory protein and identifying the most essential amino acid partial sequence that is actually needed for repressing transcription of genes.

Disclosure of the Invention

In order to attain the above objects, the present inventors have conducted concentrated studies concerning proteins having the aforementioned conserved motifs. As a result, they have found that a protein that represses the transcription of genes may have a very simple structure, and such short peptide is capable of converting a transcription factor into a transcriptional repressor. The present inventors have also found that the *Arabidopsis* protein, SUPERMAN (hereinafter it may be referred to as "SUP"), has a motif that does not match the aforementioned conserved motifs. However, such protein is capable of converting a transcription factor into a transcriptional repressor, and a chimeric gene in which a gene encoding the SUP protein is fused to a gene encoding a transcription factor can produce proteins having potent capacities for repressing transcription of genes. This has led to the completion of the present invention.

Specifically, the present invention includes the following inventions.

(1) A peptide having the amino acid sequence represented by formula (I) and capable of converting a transcription factor into a transcriptional repressor:

X1-Leu-Asp-Leu-X2-Leu-X3 (I)

wherein X1 denotes 0 to 10 amino acid residues; X2 denotes Asn or Glu; and X3 denotes at least 6 amino acid residues.

(2) A peptide having the amino acid sequence represented by formula (II) and capable of converting a transcription factor into a transcriptional repressor:

Y1-Phe-Asp-Leu-Asn-Y2-Y3 (II)

wherein Y1 denotes 0 to 10 amino acid residues; Y2 denotes Phe or Ile; and Y3 denotes at least 6 amino acid residues.

(3) A peptide having the amino acid sequence represented by formula (III) and capable of converting a transcription factor into a transcriptional repressor:

Z1-Asp-Leu-Z2-Leu-Arg-Leu-Z3 (III)

wherein Z1 denotes Leu, Asp-Leu, or Leu-Asp-Leu; Z2 denotes Glu, Gln, or Asp; and Z3 denotes 0 to 10 amino acid residues.

(4) A peptide having the amino acid sequence represented by Asp-Leu-Z4-Leu-Arg-Leu (wherein Z4 denotes Glu, Gln, or Asp) and capable of converting a transcription factor into a transcriptional repressor.

(5) A protein having any of the following amino acid sequences (a) to (d) and capable of converting a transcription factor into a transcriptional repressor:

(a) the amino acid sequence as shown in SEQ ID NO: 31;

(b) an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 31 by deletion, substitution, or addition of one or a plurality of amino acid residues;

(c) the amino acid sequence as shown in SEQ ID NO: 61; or

(d) an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 61 by deletion, substitution, or addition of one or a plurality of amino acid residues.

(6) A gene encoding a peptide having the amino acid sequence represented by formula (I) and capable of converting a transcription factor into a transcriptional repressor:

X1-Leu-Asp-Leu-X2-Leu-X3 (I)

wherein X1 denotes 0 to 10 amino acid residues; X2 denotes Asn or Glu; and X3 denotes at least 6 amino acid residues.

(7) A gene encoding a peptide having the amino acid sequence represented by formula (II) and capable of converting a transcription factor into a transcriptional repressor:

Y1-Phe-Asp-Leu-Asn-Y2-Y3 (II)

wherein Y1 denotes 0 to 10 amino acid residues; Y2 denotes Phe or Ile; and Y3 denotes at least 6 amino acid residues.

(8) A gene encoding a peptide having the amino acid sequence represented by formula (III) and capable of converting a transcription factor into a transcriptional repressor:

Z1-Asp-Leu-Z2-Leu-Arg-Leu-Z3 (III)

wherein Z1 denotes Leu, Asp-Leu, or Leu-Asp-Leu; Z2 denotes Glu, Gln, or Asp; and Z3 denotes 0 to 10 amino acid residues.

(9) A gene encoding a peptide having the amino acid sequence represented by Asp-Leu-Z4-Leu-Arg-Leu (wherein Z4 denotes Glu, Gln, or Asp) and capable of converting a transcription factor into a transcriptional repressor.

(10) A gene encoding a protein having any of the following amino acid sequences (a) to (d) and capable of converting a transcription factor into a transcriptional repressor:

(a) the amino acid sequence as shown in SEQ ID NO: 31;

(b) an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 31 by deletion, substitution, or addition of one or a plurality of amino acid residues;

(c) the amino acid sequence as shown in SEQ ID NO: 61; or

(d) an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 61 by deletion, substitution, or addition of one or a plurality of amino acid residues.

(11) Double-stranded DNA comprising a region encoding any of the above peptides or proteins (1) to (5) and having restriction enzyme sites at its both ends.

(12) A chimeric protein, wherein any of the above peptides or proteins (1) to (5) is fused to a transcription factor.

(13) A chimeric gene, wherein any of the above genes (6) to (10) is fused to a gene encoding a transcription factor.

(14) A recombinant vector comprising the chimeric gene according to (13).

(15) A transformant comprising the recombinant vector according to (14).

(16) A plant comprising the recombinant vector according to (14).

Brief Description of the Drawings

Fig. 1 shows a procedure for constructing an effector plasmid GAL4DB-RD comprising a variety of DNA fragments that are to be tested.

Fig. 2 shows the first half of the procedure for constructing a reporter gene p35S-GAL4-LUC.

Fig. 3 shows the last half of the procedure for constructing a reporter gene p35S-GAL4-LUC.

Fig. 4A shows a reporter gene and an effector plasmid. In Fig. 1 to Fig. 4A, 5XGAL4 represents the DNA-binding sequence for the GAL4 transcription factor; TATA represents a region comprising the TATA box of the CaMV 35S promoter; LUC represents a luciferase gene; CaMV 35S represents the 35S protein gene promoter derived from the cauliflower mosaic virus: GAL4DB represents a region encoding the DNA binding domain of yeast GAL4 transcription factor; and Nos represents the transcriptional terminator of the nopaline synthase gene.

Fig. 4B shows the effects of a variety of peptides fused to pGAL4DB on activity of the reporter gene (relative activity), wherein the graph on the right side shows activity of the reporter gene when the effector plasmid comprising a variety of DNA fragments is introduced (the activity of the reporter gene without the effector plasmid was set to be 100).

Fig. 5 shows a procedure for constructing the effector plasmid pGAL4DB-SUP.

Fig. 6 shows a procedure for constructing the effector plasmid pAtERF5.

Fig. 7 shows the first half of the procedure for constructing the reporter plasmid pGAL4-GCC-LUC.

Fig. 8 shows the last half of the procedure for constructing the reporter plasmid pGAL4-GCC-LUC.

Fig. 9A schematically shows the structure of 35S-GAL4-LUC that was incorporated in a plasmid as a reporter gene and the structure of SUP(D) that was incorporated as an effector gene in the transcriptional repression test.

Fig. 9B shows the results of the test for transcriptional repression activities using the SUP gene and a fragment thereof.

Fig. 10A schematically shows the structure of GAL4-GCC-LUC which was used as a reporter gene and structures of effector genes constructed by incorporating AtERF5, GAL4DB, GAL4DB-SUP, and GAL4DB175/204SUP, respectively, in the transcriptional repression test.

Fig. 10B shows the results of the transcriptional repression test using each effector plasmid.

Fig. 11 is a photograph showing the effects of the SUP gene and the ERF3 gene in repressing functions of EIN3 to activate transcription, which are investigated based on the growth level of the stem and root of a plant in the presence of an ethylene.

Fig. 12 is a photograph showing the effects of the SUP gene and the ERF3 gene in repressing functions of EIN3 to activate transcription, which are investigated based on the growth level of a plant in the presence of ethylene.

Fig. 13 is a photograph showing the effects of the SUP gene and the ERF3 gene in repressing the expression of PDF1.2, BCHN, and ERF1 genes in the presence of ethylene, which are investigated based on Northern blot hybridization using the detected mRNA representing the expression of these ethylene-inducible genes as an indicator.

Fig. 14 schematically shows the structure of the plasmid p35S::CUC1SRD for transforming *Arabidopsis thaliana*.

Fig. 15 is a photograph showing 5- to 10- days old cotyledons of a wild-type (Col-0), cuc1/cuc2 double mutant (cuc1/cuc2), and a plant transformed with p35S::CUC1SRD (35S::CUC1SRD).

Fig. 16 is a photograph showing the conditions of the stems and roots of *Arabidopsis thaliana* transformed with pEIN3SRD1 (35S::EIN3SRD1) and *Arabidopsis*

thaliana transformed with EIN3RD1 (35S::EIN3RD1) observed in the presence of ethylene.

Fig. 17 is a photograph showing the growth level of *Arabidopsis thaliana* transformed with pEIN3SRD1 (35S::EIN3SRD1) and *Arabidopsis thaliana* transformed with EIN3RD1 (35S::EIN3RD1) observed in the presence of ethylene.

Fig. 18A is a photograph of showing the seedlings of *Arabidopsis thaliana* transformed with p35S::PAP1SRDX and wild-type *Arabidopsis thaliana* that were grown on 3% sucrose-containing MS medium.

Fig. 18B is an electrophoretogram showing the expression profile of DRF gene. in the aforementioned plant by RT-PCR.

Fig. 19A is a photograph showing leaves of *Arabidopsis thaliana* transformed with p35S::AtMYB23SRDX and wild-type *Arabidopsis thaliana.*

Fig. 19B is an electrophoretogram showing the expression profile of the gene involved in the trichome development in the aforementioned plant by RT-PCR.

Hereinafter, the present invention is described in greater detail.

The present invention provides a peptide having the amino acid sequence represented by formula (I) and capable of converting a transcription factor into a transcriptional repressor:

X1-Leu-Asp-Leu-X2-Leu-X3 (I)

wherein X1 denotes 0 to 10 amino acid residues; X2 denotes Asn or Glu; and X3 denotes at least 6 amino acid residues.

In formula (I), X1 may be 0 to 10 amino acid residues, although a shorter sequence is more convenient in terms of the ease of peptide synthesis. Thus, the number of amino acid residues is preferably 10 or less, and more preferably 5 or less.

The number of amino acid residues denoted by X3 is essential, and a minimum of 6 amino acid residues was unexpectedly found to be sufficient for exhibiting the aforementioned functions. Further, X1 and X3 may be amino acids of any type. For

example, P (proline) in the aforementioned conserved motif (L/F)DLN(L/F)(X)P of peptides described in the Background Art section is not necessary for X3. X3 may be simply comprised of aligned alanines.

In contrast, a sequence consisting of LDLNL (Leu-Asp-Leu-Asn-Leu) or LDLN (Leu-Asp-Leu-Asn) does not exhibit the aforementioned functions. A sequence that was designed to have 5 or 6 amino acid residues denoted by X2 very significantly exhibits the aforementioned functions whereas the one designed to have 3 amino acid residues does not exhibit such functions.

The present invention also provides a peptide having the amino acid sequence represented by formula (II) and capable of converting a transcription factor into a transcriptional repressor:

Y1-Phe-Asp-Leu-Asn-Y2-Y3 (II)

wherein Y1 denotes 0 to 10 amino acid residues; Y2 denotes Phe or Ile; and Y3 denotes at least 6 amino acid residues.

In formula (II), Y1 may be 0 to 10 amino acid residues, although a shorter sequence is more convenient in terms of the ease of peptide synthesis. Thus, the number of amino acid residues is preferably 10 or less, and more preferably 5 or less.

A minimum of 6 amino acid residues denoted by Y3 were found to be sufficient for exhibiting the aforementioned functions. Further, Y1 and Y3 may be any amino acids.

The present invention also provides a peptide having the amino acid sequence represented by formula (III) and capable of converting a transcription factor into a transcriptional repressor:

Z1-Asp-Leu-Z2-Leu-Arg-Leu-Z3 (III)

wherein Z1 denotes Leu, Asp-Leu, or Leu-Asp-Leu; Z2 denotes Glu, Gln, or Asp; and Z3 denotes 0 to 10 amino acid residues.

In formula (III), Z3 may be 0 to 10 amino acid residues, although a shorter sequence is more convenient in terms of the ease of peptide synthesis. Thus, the number of amino acid residues is preferably 10 or less, and more preferably 5 or less.

Specific examples of Z3 include, but are not limited to, G, GFF, GFA, GYY, and AAA. A peptide represented by formula (III) has a motif DLELRL that is different from a conserved motif (L/F)DLN(L/F)(X)P of peptides as described in the Background Art section. This motif corresponds to the amino acid sequence (the 196/201 region) of the SUP protein (Asp-Leu-Glu-Leu-Arg-Leu). The total number of peptides is preferably 20 amino acids at a maximum in terms of the ease of peptide synthesis. Examples of preferable peptides include the following.

Leu-Asp-Leu-Glu-Leu-Arg-Leu,

Leu-Asp-Leu-Glu-Leu-Arg-Leu-Gly,

Leu-Asp-Leu-Glu-Leu-Arg-Leu-Ala-Ala-Ala

Leu-Asp-Leu-Glu-Leu-Arg-Leu-Gly-Phe-Ala

Asp-Leu-Asp-Leu-Glu-Leu-Arg-Leu-Gly-Phe-Ala

Leu-Asp-Leu-Asp-Leu-Glu-Leu-Arg-Leu-Gly-Phe-Ala

The peptide in the present invention that is capable of converting the transcription factor into a transcriptional repressor may comprise the minimum sequence Asp-Leu-Glu-Leu-Arg-Leu.

In the above peptide, glutamic acid (E) in the minimum sequence may be substituted with glutamine (Q) or aspartic acid (D). Peptides, such as Leu-Asp-Leu-Gln-Leu-Arg-Leu-Gly-Tyr-Tyr or Asp-Leu-Asp-Leu-Arg-Leu, have excellent effects of repressing transcriptional activities. In contrast, the sequence Leu-Glu-Leu-Arg-Leu does not have a function of transcriptional repression.

Accordingly, only 5 or 6 amino acid residues are required in the peptides represented by formulae (I) to (III) in order to convert a transcription factor into a transcriptional repressor.

The present invention provides a protein having any of the following amino acid sequences (a) to (d) and capable of converting a transcription factor into a transcriptional repressor:

(a) the amino acid sequence as shown in SEQ ID NO: 31;

(b) an amino acid sequence derived from the amino acid sequence as shown in

SEQ ID NO: 31 by deletion, substitution, or addition of one or a plurality of amino acid residues;

(c) the amino acid sequence as shown in SEQ ID NO: 61; or

(d) an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 61 by deletion, substitution, or addition of one or a plurality of amino acid residues.

The range of "one or a plurality of" in "an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 31 (or SEQ ID NO: 61) by deletion, substitution, or addition of one or a plurality of amino acid residues" is not particularly limited. For example, the number of amino acids is approximately 1 to 20, preferably 1 to 10, more preferably 1 to 7, still more preferably 1 to 5, and particularly preferably 1 to 3.

The aforementioned deletion, substitution, or addition of amino acid residues can be implemented by modifying a gene encoding the aforementioned protein in accordance with a technique conventional in the art. Mutation can be introduced to a gene by conventional techniques such as the Kunkel method or the gapped duplex method or techniques in accordance therewith. Mutation can be introduced by, for example, using a kit for introducing mutation utilizing site-specific mutagenesis (e.g., Mutant-K (Takara) or Mutant-G (Takara)) or the LA PCR *in vitro* Mutagenesis Series kit (Takara).

The SUP protein having the amino acid sequence as shown in SEQ ID NO: 31 and SUP gene are already known. The amino acid sequence (residues 195 to 199, corresponding to the nucleotide sequence (the 583/597 region)) are leucine (L)-aspartic acid (D)-leucine (L)-glutamic acid (E)-leucine (L), and a proline residue is not present downstream of this sequence toward the 3'-terminus. Instead, an amino acid sequence that is different from the motif (L/F)DLN(L/F)(X)P as mentioned in the Background Art section is present in the aforementioned sequence.

In the present invention, a protein used for converting a transcription factor into a transcriptional repressor is not limited to the one having the full-length of the amino

acid sequence as shown in SEQ ID NO: 31. The protein may be a protein or peptide comprising a partial sequence of the aforementioned amino acid sequence.

An example of a protein having such partial sequence has the amino acid sequence as shown in SEQ ID NO: 61 (the 175/204 region of the SUP protein). An example of a peptide having a partial sequence is represented by formula (III).

The present invention provides a gene encoding any of the aforementioned peptides or proteins.

The present invention also provides a chimeric protein in which any of the aforementioned peptides or proteins is fused to a transcription factor and a chimeric gene in which the gene encoding any of the aforementioned peptides or proteins is fused to a gene encoding a transcription factor. In a transformant prepared by using a recombinant vector comprising the aforementioned chimeric gene, a chimeric protein corresponding to the aforementioned chimeric gene is produced. The DNA-binding region derived from a transcription factor in this chimeric protein binds to a target gene. In this case, however, functions of a transcription factor are converted to those for repressing transcription, and transcription of the target gene is repressed. Accordingly, the target gene is not expressed.

The chimeric protein of the present invention is capable of repressing transcription of genes regardless of the type of gene. The chimeric protein of the present invention is required to bind to the target gene in order to repress its transcription. Accordingly, the gene encoding a peptide or protein (hereinafter, it may be referred to as "the gene of the present invention") is allowed to fuse with a gene encoding a DNA-binding domain of a transcription factor that binds to a specific target gene to prepare a chimeric gene, and transcription of specific target genes can be selectively repressed.

Specifically, the chimeric gene of the present invention expresses a chimeric protein in which a peptide or protein capable of converting a transcription factor into a transcriptional repressor is fused to transcription factor. The aforementioned chimeric gene specifically represses the transcription of genes to which a DNA-binding domain

derived from a transcription factor of the chimeric protein binds. When transcription of a given gene is intended to be repressed, therefore, transcription of such gene may be repressed by selecting a transcription factor that plays a key role in transcription of the aforementioned gene, ligating the gene of the present invention to the terminus or a DNA-binding domain of a gene encoding the aforementioned transcription factor to construct a chimeric gene, ligating the resulting gene to an adequate vector, and introducing the resultant into a site of an organism of interest.

A chimeric protein resulting from the chimeric gene of the present invention specifically represses the transcription of a gene to which a DNA-binding domain of the transcription factor binds. Such repression appears as a dominant trait. That is, such chimeric protein can repress functions of other transcription factors that are also involved in this gene transcription.

This is described more specifically with reference to a case where the cup-shaped cotyledon 1 (CUC1) transcription factor is used (Plant Cell, 9, 841, 1997).

CUC1 is a transcription factor that regulates apical bud formation of seedlings together with CUC2 having the same NAC domain. Only when mutation is present in both CUC1 and CUC2 genes, the cotyledon of the plant forms a cup-like shape (a cup-shaped cotyledon), and the apical meristem is not formed. In contrast, a plant having mutation in only either CUC1 or CUC2 is normal. Accordingly, CUC1 and CUC2 are known to be functionally redundant factors (Development, 126, 1563, 1999; Development, 128, 1127, 2000). When a chimeric gene, in which a gene encoding the peptide of the present invention is bound to either one of the functionally redundant CUC1 or CUC2 transcription factor genes, for example, the CUC1 gene, is allowed to express in a plant, the expressed chimeric protein can suppress transcription activity of the functionally redundant CUC2 transcription factor as well as that of the CUC1 transcription factor. That is, it can repress the expression of genes regulated by the CUC1 transcription factor. In such a case, the cotyledon of the plant forms a cup-like shape, which is a trait of a cuc1/cuc2 double mutant (a cup-shaped cotyledon), and the apical meristem is not formed. In the Example 5 below, a chimeric gene was

constructed, wherein the gene encoding the DLDLELRLGFA peptide of the present invention (this peptide is referred to as "SRD") had been allowed to fuse with the CUC1 gene (Fig. 14), and *Arabidopsis thaliana* was transformed with the chimeric gene. This demonstrates that the transgenic plant takes on a cup-like shape indicating a phenotype of a cuc1/cuc2 double mutant (a cup-shaped cotyledon) (Fig. 15, right). Formation of the apical meristem is not observed as with the case of the deficiencies of STM gene which regulates the formation of the apical meristem regulated by the CUC1 transcription factor. This indicates that the CUC1 transcription factor capable of activating transcription was functionally converted to a transcriptional repressor via fusion with the DLDLELRLGFA peptide of the present invention. The aforementioned further indicates that the above peptide suppresses not only activity of CUC1 transcription factor, but also preferentially suppresses activity of the CUC2 transcription factor which is functionally redundant with CUC1, and represses expression of genes located downstream.

As shown from the foregoing, the peptide of the present invention and a gene encoding the peptide are capable of converting any transcription factor into a transcriptional repressor and are also capable of suppressing activity of other transcription factors that are functionally redundant.

In many cases, a plant has a plurality of functionally redundant transcription factors as demonstrated with reference to CUC. The transcriptional repressor, functions of which had been converted by the present invention, appears as a dominant trait. The present invention is, therefore, very useful since functions of transcription factors that were not elucidated by conventional single-gene knockout technology can be analyzed, and it can be effectively applied to plants having amphidiploid genomes such as wheat.

As mentioned above, the chimeric gene of the present invention produces a chimeric protein corresponding thereto, and this chimeric protein is allowed to bind to the target gene. Thus, transcription of the target gene is repressed. Accordingly, this

chimeric protein is separately synthesized and the resultant may be directly introduced into a site in an organism where the target genes are expressed.

This chimeric protein may be synthesized by a conventional technique of genetic engineering. For example, the chimeric gene may be incorporated into an adequate vector, microorganisms may be transformed using the same, and the transgenic microorganisms may be cultured. Thus, a large amount of the chimeric proteins can be synthesized.

The site where the gene of the present invention is fused to a transcription factor is located downstream of the region encoding the DNA-binding domain in the transcription factor. When the gene of the present invention is intended to be inserted in a gene encoding a transcription factor, this insertion involves laborious operations such as cleavage of the gene encoding a transcription factor or ligation and recombination of the gene of the present invention. Accordingly, it is convenient to simply bind the gene of the present invention to the terminus located downstream of the region encoding the transcription factor protein. This is one of the advantages of the present invention.

The gene of the present invention may have any nucleotide sequence as long as it encodes a peptide having any of the amino acid sequences represented by formulae (I) to (III) or a protein having the amino acid sequence as shown in SEQ ID NO: 31 or 61. The gene of the present invention may have a site where it is fused to the gene encoding a transcription factor. When the amino acid reading frame of the gene of the present invention is not in-frame with the reading frame of the gene encoding the transcription factor, the gene should be designed to have a reading frame to be in-frame with that of interest. Thus, the gene may have additional nucleotide sequences.

In the present invention, gene transcription may be repressed by directly introducing the aforementioned chimeric protein in an organism. When breed improvement in plant is intended, for example, transcription of a specific gene must be constantly repressed to repress expression thereof. Accordingly, it is more effective to ligate a gene encoding the aforementioned chimeric protein to an adequate vector and

transform plants and the like using the resulting recombinant vector. This enables a gene encoding a chimeric protein to be constantly expressed in a plant, and the resulting chimeric protein continuously represses transcription of genes.

This mechanism of repressing transcription is described in greater detail with reference to a case where the *Arabidopsis thaliana* ethylene-insensitive 3 gene (hereafter referred to as the "EIN3 gene") is used as a transcription factor. The sequence of this EIN3 gene and that of a protein produced therefrom are shown in SEQ ID NO: 52.

The EIN3 protein factor, which is the EIN3 gene product, serves as a transcription factor and is a factor in ethylene signal transduction mediating biological activities induced by phytohormone ethylene, such as morphological changes of the etiolated seedling (the triple response), inhibition of elongation, and expression of an ethylene responsive gene.

A gene fragment encoding the peptide or protein according to the present invention is fused to a region encoding the DNA-binding domain of the EIN3 gene to prepare a chimeric gene. The resultant is ligated to, for example, a vector for plant transformation having the cauliflower mosaic virus 35S promoter, and *Arabidopsis thaliana* is transformed using this recombinant vector. Wild-type *Arabidopsis thaliana* exhibits morphological changes in the etiolated seedling (the triple response) and inhibition of elongation in the presence of ethylene or its precursor 1-aminocyclopropane-D-carboxylic acid. In the case of transgenic *Arabidopsis thaliana*, however, ethylene-responsive biological activities thereof are significantly suppressed. Accordingly, the gene of the present invention is capable of converting the function of EIN3 for activating transcription to that for repressing transcription.

In the present invention, examples of a transcription factor that is converted into a transcriptional repressor and a gene thereof include, but are not limited to, the aforementioned EIN3 and a gene thereof, yeast GAL4, ERF4, CBF1, ERF2, EREB1, CUC1, and CUC2 proteins and genes thereof. Transcription factors of animals, plants, and microorganisms and genes thereof can be extensively employed.

Best Modes for Carrying out the Invention

The present invention is hereafter described with reference to the following examples, although the technical scope of the present invention is not limited thereto.

In Example 1, (i) an effector plasmid in which a variety of synthesized gene fragments fused to the encoding region of the DNA-binding domain of the yeast GAL4 transcription factor was ligated to the downstream region of the cauliflower mosaic virus 35S promoter, which functions in plant cells, and (ii) a reporter gene comprising the luciferase gene in which the enhancer region of the cauliflower mosaic virus 35S promoter, the GAL4 protein-binding DNA sequence, and the TATA region of the cauliflower mosaic virus 35S promoter are ligated to the promoter region were constructed. These effector plasmid and reporter gene were co-introduced into *Arabidopsis thaliana* leaves using a particle gun, and activity of the luciferase gene, i.e., the reporter gene, was assayed to investigate the repression activity of transcription of the synthesized gene fragments.

In Example 2, the transcriptional repression activity of the gene encoding a protein having the full-length SUP amino acid sequence and the gene encoding the partial amino acid sequence (the 175/204 region) of the SUP protein was investigated based on the assay of luciferase activity of the reporter gene.

In Example 3, the activity of the gene encoding the partial SUP protein having the amino acid sequence (the 175/204 region) of SUP for repressing transcriptional function of EIN3 was investigated in plants.

In Example 4, the activity of the gene encoding the partial ERF3 protein having the amino acid sequence (the 191/225 region) of ERF3 for repressing transcriptional function of EIN3 was investigated in plants.

In Example 5, a gene fragment encoding DLDLELRLGFA (SUPERMAN repression domain (SRD), residues 194-204) was fused to a transcription factor CUC1, and the resultant was ligated to the downstream region of the cauliflower mosaic virus 35S promoter to construct a plasmid for transformation, *Arabidopsis thaliana* was transformed using the aforementioned plasmid, and morphological changes of the

cotyledon after germination were observed. Thus, effects of the aforementioned gene fragment in repressing the expression of the genes for CUC1, and for CUC2 that is functionally redundant with CUC1, were investigated

In Example 6, a gene encoding LDLELRLGFA (SUPERMAN repression domain (SRD1), residues 195-204) and LDLNLAPPMEF (ERF3 repression domain (RD1), residues 215-225) was fused to a plant transcription factor EIN3, *Arabidopsis thaliana* was transformed in the same manner, and morphological changes of relevant plants in the presence of ethylene were observed. Thus, effects of the aforementioned gene fragment in repressing transcription of the target gene for EIN3 were inspected.

In Example 7, a chimeric repressor (35S::PAP1SRDX) prepared by applying a peptide (SRDX) consisting of 12 amino acid residues represented by the amino acid sequence LDLDLELRLGFA to the carboxyl terminus of the production-of-anthocyanin-pigment 1 transcription factor (PAP1) (Borevitz J. O., Xia Y., Blount J., Dixon R. A. & Lamb C., Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis, Plant Cell 12, 2383, 2000)) was introduced into *Arabidopsis thaliana* to produce the transgenic plants. The effects of repressing the transcription of an anthocyanin-synthesizing gene in the plants were inspected.

In Example 8, a chimeric repressor (35S::AtMYB23SRDX) prepared by applying a peptide (SRDX) consisting of 12 amino acid residues represented by the amino acid sequence LDLDLELRLGFA to the carboxyl terminus of the AtMYB23 transcription factor (Kirik V., Schnittger A., Radchuk V., Adler K., Hulskamp M., & Baumlein H., Ectopic expression of the *Arabidopsis* AtMYB23 gene induces differentiation of trichome cells, Dev Biol. 235, 366 (2001); a conserved MYB regulator of phenylpropanoid biosynthesis, Plant Cell 12, 2383 (2000)) was introduced to *Arabidopsis thaliana* to produce the transgenic plants. The effects of repressing the transcription of genes that regulate trichome generation were inspected.

In Example 9, a repression activity of transcription was tested using tobacco leaf and petunia.

(Example 1) Identification of peptide that serves as a repression domain

(1) Construction of effector plasmid pGAL4DB-RD (Fig. 1)

Plasmid pBI221 (Clontech, U.S.A.) was cleaved with restriction enzymes *Xho*I and *Sac*I and blunt-ended with T4 polymerase. Thereafter, the GUS gene was removed by agarose gel electrophoresis to obtain a fragment of 35S-Nos plasmid DNA comprising the cauliflower mosaic virus 35S promoter (hereafter referred to as "CaMV 35S") and a transcription terminator of the nopaline synthase gene (Nos terminator, hereafter referred to as "Nos-ter").

The pAS2-1 vector (Clontech) was digested with a restriction enzyme *Hin*dIII, a 748 bp DNA fragment encoding a DNA-binding domain of the yeast GAL4 protein (hereafter this fragment (the 1/147 region) is referred to as "GAL4DBD") was isolated by agarose gel electrophoresis, and the isolated fragment was blunt-ended with T4 DNA polymerase. This DNA fragment comprising the GAL4DBD-encoding region was inserted in the blunt-ended site between the 35S promoter and the Nos terminator of the aforementioned 35S-Nos DNA fragment, and a clone in which the ORFs of the DNA-binding domain of the yeast GAL4 protein are oriented in the forward direction relative to the 35S promoter was selected to construct a p35S-GAL4DBD vector.

Two DNA strands encoding a peptide to be tested, which had been designed to have a reading frame to be in-fame with the amino acid reading frame of GAL4DBD, were synthesized. The nucleotide sequences of the synthesized DNA and the amino acid sequences encoded thereby are shown below.

ERF3RD (214/225)

Amino acid sequence: DLDLNLAPPMEF (SEQ ID NO: 1)

5'-CGATCTTGATCTTAACCTTGCTCCACCTATGGAATTTTGAG-3' (SEQ ID NO: 2)

5'-TCGACTCAAAATTCCATAGGTGGAGCAAGGTTAAGATCAAGATCG-3' (SEQ ID NO: 3)

3 RD1

Amino acid sequence: LDLNLAPPMEF (SEQ ID NO: 4)

5'-CCTTGATCTTAACCTTGCTCCACCTATGGAATTTTGAG-3' (SEQ ID NO: 5)

5'-TCGACTCAAAATTCCATAGGTGGAGCAAGGTTAAGATCAAGG-3' (SEQ ID NO: 6)

3 RD2

Amino acid sequence: LDLNLAAAAAA (SEQ ID NO: 7)

5'-CCTTGATCTTAACCTTGCTGCTGCTGCTGCTTGAG-3' (SEQ ID NO: 8)

5'-TCGACTCAAGCAGCAGCAGCAGCAAGGTTAAGATCAAGG-3' (SEQ ID NO: 9)

Min-LDLN

Amino acid sequence: LDLN (SEQ ID NO: 10)

5'-CCTGGATCTAAATTAAG-3' (SEQ ID NO: 11)

5'-TCGACTTAATTTAGATCCAGG-3' (SEQ ID NO: 12)

Min-LDLNL

Amino acid sequence: LDLNL (SEQ ID NO: 13)

5'-CCTGGATCTAAATCTGTAAG-3' (SEQ ID NO: 14)

5'-TCGACTTACAGATTTAGATCCAGG-3' (SEQ ID NO: 15)

SRD1

Amino acid sequence: LDLELRLGFA (SEQ ID NO: 16)

5'-CCTGGATCTAGAACTCCGTTTGGGTTTCGCTTAAG-3' (SEQ ID NO: 17)

5'-TCGACTTAAGCGAAACCCAAACGGAGTTCTAGATCCAGG-3' (SEQ ID NO: 18)

SRD2

Amino acid sequence: LDLELGFA (SEQ ID NO: 19)

5'-CCTGGATCTAGAACTCGGTTTCGCTTAAG-3' (SEQ ID NO: 20)

5'-TCGACTTAAGCGAAACCGAGTTCTAGATCCAGG-3' (SEQ ID NO: 21)

LELDL

Amino acid sequence: LELDLAAAAAA (SEQ ID NO: 22)

5'-ACTGGAACTAGATCTAGCTGCAGCTGCAGCTGCTTAAG-3' (SEQ ID NO: 23)

5'-TCGACTTAAGCAGCTGCAGCTGCAGCTAGATCTAGTTCCAGT-3' (SEQ ID NO: 24)

Amino acid sequence: LELRLAAAAAA (SEQ ID NO: 80)

5'-ACTAGAACTCCGTTTGGCTGCCGCAGCGGCTGCATAATGAG-3' (SEQ ID NO: 81)

5'-TCGACTCATTATGCAGCCGCTGCGGCAGCCAAACGGAGTTCTAGT-3' (SEQ ID NO: 82)

Amino acid sequence: DLELRL (SEQ ID NO: 83)

5'-AGATCTAGAACTCCGTTTGTAATGAG-3' (SEQ ID NO: 84)

5'-TCGACTCATTACAAACGGAGTTCTAGATCT-3' (SEQ ID NO: 85)

Amino acid sequence: LDLQLRLGYY (SEQ ID NO: 86)

5'-ACTGGATCTACAACTCCGTTTGGGTTATTACTAATGAG-3' (SEQ ID NO: 87)

5'-TCGACTCATTAGTAATAACCCAAACGGAGTTGTAGATCCAG-3' (SEQ ID NO: 88)

Amino acid sequence: LDLELRL (SEQ ID NO: 89)

5'-ACTGGATCTAGAACTCCGTTTGTAATGAG-3' (SEQ ID NO: 90)

5'-TCGACTCATTACAAACGGAGTTCTAGATCCAG T-3' (SEQ ID NO: 91)

Amino acid sequence: LDLELAAAAAA (SEQ ID NO: 92)

5'-ACTGGATCTAGAACTCGCTGCCGCAGCGGCTGCATAATGAG-3' (SEQ ID NO:

93)

5'-TCGACTCATTATGCAGCCGCTGCGGCAGCGAGTTCTAGATCCAGT-3' (SEQ ID NO: 94)

Amino acid sequence: LDLELRLAAA (SEQ ID NO: 95)

5'-ACTGGATCTAGAACTCCGTTTGGCTGCCGCATAATGAG-3' (SEQ ID NO: 96)

5'-TCGACTCATTATGCGGCAGCCAAACGGAGTTCTAGATCCAGT-3' (SEQ ID NO: 97)

Amino acid sequence: LELDLAAAAAA (SEQ ID NO: 98)

5'-CCTTGAGCTTGATCTTGCTGCTGCTGCTGCTTGAG-3' (SEQ ID NO: 99)

5'-TCGACTCAAGCAGCAGCAGCAGCAAGATCAAGCTCAAGG-3' (SEQ ID NO: 100)

Amino acid sequence: LDLELRLG (SEQ ID NO: 101)

5'-CCTGGATCTAGAACTCCGTGGTTAAG-3' (SEQ ID NO: 102)

5'-TCGACTTAACCACGGAGTTCTAGATCCAGG -3' (SEQ ID NO: 103)

Amino acid sequence: LELRL (SEQ ID NO: 104)

5'-TCTA GAA CTC CGT TTG TAA TGAG-3' (SEQ ID NO: 105)

5'-TCGACTCA TTA CAA ACG GAG TTC TAG A-3' (SEQ ID NO: 106)

Amino acid sequence: FDLNFAPLDCV (SEQ ID NO: 107)

5'-ATTCGATCTTAATTTTGCACCGTTGGATTGTGTTTAAG-3' (SEQ ID NO: 108)

5'-TCGACTCATTAAACACAATCCAACGGTGCAAAATTAAGATCGAAT-3' (SEQ ID NO: 109)

Amino acid sequence: FDLNIFPPIPEF (SEQ ID NO: 110)

5'-GTTTGACCTCAACATCCCTCCGATCCCTGAATTCTAAG-3' (SEQ ID NO: 111)

5'-TCGACTTAGAATTCAGGGATCGGAGGGATGTTGAGGTCAAAC-3' (SEQ ID NO: 112)

Amino acid sequence: FQFDLNFPPLDCV (SEQ ID NO: 113)

5'-CTTTCAATTCGATCTTAATTTTCCACCGTTGGATTGTGTTTAAG-3' (SEQ ID NO: 114)

5'-TCGACTTAAACACAATCCAACGGTGGAAAATTAAGATCGAATTGAAAG-3' (SEQ ID NO: 115)

Amino acid sequence: DLDLRL (SEQ ID NO: 116)

5'-ACTGGATCTAGATCTCCGTTTGTAATGAG-3' (SEQ ID NO: 117)

5'-TCGACTCATTACAAACGGAGATCTAGATCCAGT-3' (SEQ ID NO: 118)

Each of a DNA fragment encoding these peptides was incorporated into the plasmid p35S-GAL4DBD that had been previously digested with restriction enzymes, *Sma*I and *Sal*I, to construct the effector plasmid pGAL4DB-RD.

(2) Construction of reporter gene

(2-1) Construction of reporter gene pGAL4-LUC (Fig. 2)

Plasmid pUC18 was digested with restriction enzymes, *Eco*RI and *Sst*I. Plasmid pBI221 (Clontech) was digested with restriction enzymes, *Eco*RI and *Sst*I, and a 270 bp DNA fragment comprising the nopaline synthase terminator (Nos-ter) region was isolated by agarose gel electrophoresis. The resulting fragment was inserted into the *Eco*RI-*Sst*I site of the plasmid pUC18 that had been digested with restriction enzymes *Eco*RI and *Sst*I. Complementary strands DNA 1: AGCTTAGATCTGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAG AGGACACGCTG (SEQ ID NO: 25) and DNA 2: GATCCAGCGTGTCCTCTCCAAATGAAATGAACTTCCTTATATAGAGGAAGGGT CTTGCAGATCTA (SEQ ID NO: 26) comprising the TATA box of the cauliflower mosaic virus 35S promoter were synthesized.

The synthesized DNA was incubated at 90°C for 2 minutes, further incubated at 60°C for 1 hour, and then allowed to stand at room temperature (25°C) for 2 hours for annealing to form double-stranded DNA. The plasmid pUC18 comprising Nos-ter was digested with restriction enzymes, *Hin*dIII and *Bam*HI. The synthesized double-stranded DNA was inserted into the *Hin*dIII-*Bam*HI site of pUC18 to construct a plasmid comprising the TATA-box and Nos-ter.

This plasmid was digested with a restriction enzyme, *Sst*I and then blunt-ended with T4 DNA polymerase.

The plasmid vector PGV-CS2 (Toyo Ink Mfg. Co., Ltd.) having the firefly luciferase gene (LUC) was digested with restriction enzymes, *Xba*I and *Nco*I and then blunt-ended with T4 DNA polymerase. Thereafter, a 1.65 kb DNA fragment comprising the luciferase gene was isolated and purified by agarose gel electrophoresis. This DNA fragment was inserted into the aforementioned plasmid comprising the TATA box and the Nos-terminator to construct the reporter gene pTATA-LUC.

The plasmid pG5CAT (Clontech) having 5 repeats of the yeast GAL4 DNA-binding domain was digested with restriction enzymes, *Sma*I and *Xba*I and blunt-ended with T4 DNA polymerase. Thereafter, a DNA fragment comprising 5 repeats of the GAL4 DNA-binding domain was purified by agarose gel electrophoresis. The TATA-LUC vector was digested with a restriction enzyme, *Bgl*II and blunt-ended with T4 DNA polymerase. The blunt-ended DNA fragment comprising 5 repeats of the GAL4 DNA-binding domain was inserted into this site, and a clone in which the GAL4 DNA-binding domain is oriented in the forward direction was selected from among the obtained plasmids to construct the reporter gene pGAL4-LUC (see Fig. 2).

(2-2) Construction of p35S-GAL4-LUC (Fig. 3)

PCR was carried out using the plasmid pBI121 as a template and using the 5'-primer: CGCCAGGGTTTTCCCAGTCACGAC (SEQ ID NO: 27) and the 3'-primer: AAGGGTAAGCTTAAGGATAGTGGGATTGTGCGTCATC (SEQ ID NO: 28). Thus, a DNA fragment comprising the CaMV 35S promoter domain (from -800 to -46) was obtained. After the digestion with the restriction enzyme, *Hin*dIII, a 760 bp DNA

fragment comprising the CaMV 35S promoter domain (from -800 to -46) was isolated by agarose gel electrophoresis. This *Hin*dIII-CaMV35S fragment was inserted into the reporter gene pGAL4-LUC that had been previously digested with the restriction enzyme, *Hin*dIII, and a clone in which DNA of the CaMV 35S promoter is oriented in the forward direction was selected to construct the reporter gene p35S-GAL4-LUC (see Fig. 3).

(3) Construction of reference gene

The cassette vector pRL-null (Promega) having the *Renilla* luciferase gene was cleaved with restriction enzymes *Nhe*I and *Xba*I and blunt-ended with T4 DNA polymerase. Thereafter, a 948 bp DNA fragment comprising the *Renilla* luciferase gene was purified by agarose gel electrophoresis. This DNA fragment was inserted into a region in the pBI221 vector from which the GUS gene used for constructing the effector plasmid had been removed. A clone in which the *Renilla* luciferase gene is oriented in the forward direction was selected from among the obtained plasmids (construction of pPTRL).

(4) Method for assaying of the activity of reporter gene

The reporter gene and the effector plasmid were introduced into *Arabidopsis thaliana* by the particle gun method, and effects of the effector plasmid were analyzed by assaying activity of the reporter gene.

(5) Gene introduction using particle gun

Gold grains (510 mg, diameter 1mm, Bio-Rad) were coated with 1.2 mg of DNA of the reporter gene p35S-GAL4-LUC prepared above, 1.2 mg of DNA of the effector plasmid pGAL4DB-RD prepared above, and 0.32 mg of the reference gene plasmid. *Arabidopsis thaliana* leaves (7 leaves) of 21 day-old were laid out a water-moistened filter paper in a 9-cm petri dish, and DNA was introduced therein using the PDS 1000/HE device for particle bombardment (Bio-Rad). The leaves were allowed to stand at 22°C for 6 hours in a well-lit place, and the activity of the reporter gene was then assayed.

(6) Assay of luciferase activity

The *Arabidopsis thaliana* leaves that had been allowed to stand for 6 hours were grinded in liquid nitrogen, suspended in 200 μl of the Passive Lysis Buffer from the Dual-Luciferase® Reporter Assay System (Promega), and then centrifuged to recover the supernatant. This cell extract (20 μl) was mixed with 100 μl of the assay buffer attached to the Dual-Luciferase® Reporter Assay System (Promega), and luciferase activity was assayed using a luminometer (TD 20/20, Turner Design). Activity of the firefly luciferase and that of *Renilla* luciferase were assayed by measuring luminescence over 10 seconds in the integral mode in accordance with the instructions of the assay kit. A value indicating reference gene activity was divided by a value indicating reporter gene activity, and the relative value thereof, i.e., the relative luciferase activity, was determined as a measured value. Each type of effector plasmid was subjected to a transient assay for three times, and the mean and the standard deviation were determined. The relative activity value of the reporter gene p35S-GAL4-LUC without the effector plasmid was set to be 100, and effects of the effector plasmid were analyzed based on variations in values of the reporter gene activity when the effector plasmid was co-expressed in cells. Specifically, if the value indicating reporter gene activity decreases upon introduction of the reporter gene p35S-GAL4-LUC and the effector plasmid pGAL4DB-RD comprising DNA encoding each peptide sequence incorporated therein, such decrease indicates that the peptide has the effect of repressing the activity of the reporter gene (repression activity). When the reporter gene activity was measured and the relative activity value of the reporter gene p35S-GAL4-LUC became 100 or smaller, the effector plasmid introduced was determined to have a repression activity

(7) Identification of repression domain

Fig. 4A shows the structure of the reporter gene and that of the effector plasmid. Fig. 4B and Table 1 below show the results of assaying of activity of reporter gene.

(Table 1)

Identification of peptide	Peptide sequence	Relative value (%)

ERF3RD(214/225)	DLDLNLAPPMEF	15.0
3RD1	LDLNLAPPMEF	14.6
3RD2	LDLNLAAAAAA	17.5
SRD1	LDLELRLGFA	2.0
SRD2	LDLELGFA	221
LELDL	LELDLAAAAAA	196
Min-LDLN	LDLN	153
Min-LDLNL	LDLNL	150
	LELRLAAAAAA	130.6
	DLELRL	8.9
	LDLQLRLGYY	3.8
	LDLELRL	4.5
	LDLELAAAAAA	72.5
	LDLELRLAAA	6.9
	LELDLAAAAAA	196.0
	LDLELRLG	8.9
	LELRL	101.5
	FDLNFAPLDCV	17.5
	FDLNIFPPIPEF	16.0
	FQFDLNFPPLDCV	10.9
	DLDLRL	9.2
Control	GAL4DB alone	100

According to the above results, activity of reporter gene of a peptide comprising LDL(N/E)L or FDLN(F/I) and at least 6 amino acid residues at its C-terminus or a peptide comprising DL(E/Q/D)LRL decreases by 85% to 98%, in comparison with that of the reporter gene (containing no effector plasmid, i.e., the control). This demonstrates that the aforementioned peptides serve as functional peptides capable of repressing transcriptions of genes.

The control p35S-GAL4DBD, which does not contain the coding sequence for the peptide, did not effect the activity of the reporter gene. This indicates that the aforementioned peptide bound to the GAL4 DNA-binding domain serves as a repressor of transcription.

(Example 2) Repression of transcription by SUP gene-containing effector plasmid

(1) Isolation of SUP gene

The nucleotide sequence of the SUP gene has already been reported. Oligonucleotides having sequences corresponding to the sequences on the 5'-side and the 3'-side of the protein-encoding region of the SUP gene of *Arabidopsis thaliana* were

synthesized, and PCR was carried out using the resulting sequences as primers and using the TAC clone:K14B15 (assigned from the Kazusa DNA Research Institute) comprising the SUP gene as a template. The DNA fragment comprising the protein-encoding region of the SUP gene was isolated. The total nucleotide sequence was determined, and it was confirmed to be the protein-encoding region of the SUP gene that had been already reported. The conditions for the aforementioned PCR was as follows: 25 cycles each consisting of denaturation at 94°C for 1 minute; annealing at 47°C for 2 minutes; and elongation at 74°C for 1 minute.

(2) Construction of effector plasmid

(2-1) Construction of effector plasmid, pGAL4DB-SUP, comprising the full-length protein-encoding region of the SUP gene (Fig. 5)

Plasmid pBI221 (Clontech, U.S.A.) was cleaved with restriction enzymes, *Xho*I and *Sac*I, and the GUS gene was removed by agarose gel electrophoresis and blunt-ended with T4 polymerase, to obtain a fragment of 35S-Nos plasmid DNA comprising CaMV 35S and Nos-ter.

The pAS2-1 vector (Clontech) was digested with a restriction enzyme, *Hin*dIII, GAL4DBD, i.e., a transcription activator of yeast, was isolated by agarose gel electrophoresis, and the isolated fragment was blunt-ended with T4 DNA polymerase. This DNA fragment comprising the GAL4DBD-encoding region was inserted in the blunt-ended site between the 35S promoter and the Nos terminator of the aforementioned 35S-Nos DNA fragment, and a clone in which the ORFs of the DNA-binding domain of the yeast GAL4 protein are oriented in the forward direction to the 35S promoter was selected to construct a p35S-GAL4DBD vector.

PCR was carried out using the 5'-primer 1: GATGGAGAGATCAAACAGC (SEQ ID NO: 29, bound to the nucleotide sequence (the 1/18 region) of the SUP gene) of the SUP gene and the 3'-primer 2: GATAAAGTTATTACCGTCGACTTAAGCGAAAC (SEQ ID NO: 30, bound to the nucleotide sequence (the 602/641 region) of the SUP gene) having a restriction enzyme, *Sal*I site, each of which had been designed to have the reading frame to be in-frame with that of GAL4DBD, to amplify the total

protein-encoding region of the SUP gene (SEQ ID NO: 31, the 1/204 amino acid sequence) to obtain a DNA fragment. The conditions for PCR was as follows: 25 cycles each consisting of denaturation at 94°C for 1 minute; annealing at 47°C for 2 minutes; and elongation at 74°C for 1 minute. Hereafter, all PCRs were carried out under the same conditions. The resulting DNA fragment was digested with a restriction enzyme, *Sal*I, and the DNA fragment of interest was isolated by agarose gel electrophoresis. This SUP-encoding DNA fragment was incorporated into the plasmid p35S-GAL4DB that had been previously digested with restriction enzymes, *Sma*I and *Sal*I to construct the effector plasmid pGAL4DB-SUP.

(2-2) Construction of effector plasmid pGAL4DB-175/204SUP comprising the amino acid sequence (the 175/204 region) of SUP

PCR was carried out using the 5'- primer 3: GAATGATGAAATCATCAG (SEQ ID NO: 32, bound to the nucleotide sequence (the 522/539 region) of the SUP gene) and the 3'-primer 2: GATAAAGTTATTACCGTCGACTTAAGCGAAAC (SEQ ID NO: 30, bound to the nucleotide sequence (the 602/641 region) of the SUP gene) having the restriction enzyme, *Sal*I site, each of which had been designed to have the reading frame to be in-frame with that of GAL4DBD, to obtain a DNA fragment comprising a nucleotide sequence (the 523/612 region) corresponding to the region encoding the amino acid sequence (the 175/204 region) of SUP. This DNA fragment was digested with a restriction enzyme, *Sal*I, the DNA fragment of interest was isolated by agarose gel electrophoresis, and the nucleotide sequence thereof was determined. A DNA fragment (the 523/612 region) encoding the amino acid sequence (the 175/204 region) of SUP was incorporated into the plasmid 35S-GAL4DBD that had been previously digested with restriction enzymes, *Sma*I and *Sal*I, to construct the effector plasmid pGAL4DB-175/204SUP.

(2-3) Construction of control effector plasmid (Fig. 6)

PCR was carried out using the clone pAtERF5 comprising *Arabidopsis thaliana* AtERF5cDNA as a template and using the 5'-primer 4: CATGGCGACTCCTAACGAAGTATCTGCAC (SEQ ID NO: 33) and the 3'-primer 5:

ATCGTTCAAAAACTCAAGGCTAACTAATCAACAACGGTC (SEQ ID NO: 34) to amplify the full-length protein-encoding region of AtERF5. This DNA fragment was cloned into the aforementioned blunt-ended 35S-Nos plasmid fragment to construct the effector plasmid p35S-AtERF5.

Separately, the effector plasmid pGAL4DB was constructed in the same manner as described above, except that the SUP gene and 175/204 SUP were not used.

(3) Construction of reporter gene

Two types of reporter genes, p35S-GAL4-LUC and pGAL4-GCC-LUC, were constructed in the following procedure.

(3-1) Construction of p35S-GAL4-LUC (Fig. 2 and Fig. 3)

a. Construction of pGAL4-LUC (Fig. 2)

Plasmid pUC18 was digested with restriction enzymes, *Eco*RI and *Sst*I. Separately, plasmid pBI221 (Clontech) was digested with restriction enzymes, *Eco*RI and *Sst*I, and a 270 bp DNA fragment comprising the nopaline synthase terminator (Nos-ter) region was isolated by agarose gel electrophoresis. The resulting fragment was inserted into the *Eco*RI-*Sst*I site of the plasmid pUC18 that had been digested with restriction enzymes *Eco*RI and *Sst*I. Subsequently, complementary strands of DNA 1: AGCTTAGATCTGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAG AGGACACGCTG (SEQ ID NO: 35) and DNA 2: GATCCAGCGTGTCCTCTCCAAATGAAATGAACTTCCTTATATAGAGGAAGGGT CTTGCAGATCTA (SEQ ID NO: 36) comprising the TATA box of the cauliflower mosaic virus 35S promoter were synthesized.

The synthesized DNA was incubated at 90°C for 2 minutes, further incubated at 60°C for 1 hour, and then allowed to stand at room temperature (25°C) for 2 hours for annealing to prepare double-stranded DNA. The plasmid pUC18 comprising Nos was digested with restriction enzymes, *Hin*dIII and *Bam*HI. The synthesized double-stranded DNA was inserted into the *Hin*dIII-*Bam*HI site of pUC18 to construct a plasmid comprising the TATA-box and Nos-ter.

This plasmid was digested with a restriction enzyme, *Sst*I and then blunt-ended with T4 DNA polymerase.

Separately, the plasmid vector PGV-CS2 (Toyo Ink Mfg. Co., Ltd.) having the firefly luciferase gene (LUC) was digested with restriction enzymes, *Xba*I and *Nco*I and then blunt-ended with T4 DNA polymerase. Thereafter, a 1.65 kb DNA fragment comprising the luciferase gene was isolated and purified by agarose gel electrophoresis. This DNA fragment was inserted into the aforementioned plasmid comprising the TATA box and the Nos-terminator to construct the reporter gene pTATA-LUC.

The plasmid pG5CAT (Clontech) having 5 repeats of the yeast GAL4 DNA-binding domain was digested with restriction enzymes, *Sma*I and *Xba*I and blunt-ended with T4 DNA polymerase. Thereafter, a DNA fragment comprising 5 repeats of the GAL4 DNA-binding domain was purified by agarose gel electrophoresis. The TATA-LUC vector was digested with a restriction enzyme, *Bgl*II and blunt-ended with T4 DNA polymerase. The blunt-ended DNA fragment comprising 5 repeats of the GAL4 DNA-binding domain was inserted into this restriction site, a clone in which the GAL4 DNA-binding domain is oriented in the forward direction was selected from among the obtained plasmids to construct the reporter gene pGAL4-LUC (see Fig. 2).

b. Construction of p35S-GAL4-LUC (Fig. 3)

PCR was carried out using the plasmid pBI121 as a template and using the 5'-primer 6: CGCCAGGGTTTTCCCAGTCACGAC (SEQ ID NO: 37) and the 3'-primer 7: AAGGGTAAGCTTAAGGATAGTGGGATTGTGCGTCATC (SEQ ID NO: 38). Thus, a DNA fragment comprising the CaMV 35S promoter domain (from -800 to -46) was obtained. After the digestion with the restriction enzyme, *Hin*dIII, a 760 bp DNA fragment comprising the CaMV 35S promoter domain (from -800 to -46) was isolated by agarose gel electrophoresis. This *Hin*dIII fragment was inserted into the reporter gene pGAL4-LUC that had been previously digested with the restriction enzyme, *Hin*dIII, and a clone in which DNA of the CaMV 35S promoter is oriented in the forward direction was selected to construct the reporter gene p35S-GAL4-LUC (see Fig. 3).

(3-2) Construction of pGAL4-GCC-LUC (Fig. 7 and Fig. 8)

Complementary strands (shown below) of a 45 bp DNA fragment comprising 4 GCC box sequences (AGCCGCC) were synthesized. The synthesized strands were incubated at 70°C for 15 minutes and allowed to stand at room temperature for 60 minutes for annealing to prepare double-stranded DNA.

5'-GATCAGCCGCCGATCAGCCGCCGATCAGCCGCCGATCAGCCGCC-3'
(SEQ ID NO: 39)

3'-TCGGCCGGCTAGTCGGCGGCTAGTCGGCGGCTAGTCGGCGGGATC-5'
(SEQ ID NO: 40)

This 45 bp DNA fragment was mixed with the aforementioned TATA-LUC vector that had been previously digested with a restriction enzyme *Bgl*II at a molar ratio of 1:1, ligation was carried out using T4 ligase, and a clone in which a GCC box-containing DNA fragment is oriented in the forward direction was selected to construct the plasmid pGCC-LUC. This plasmid was digested with a restriction enzyme, *Bgl*II and blunt-ended with T4 DNA polymerase (see Fig. 7).

The plasmid pG5CAT (Clontech) having 5 repeats of the yeast GAL4 DNA-binding domain was digested with restriction enzymes, *Sma*I and *Xba*I and blunt-ended with T4 DNA polymerase. Thereafter, a DNA fragment comprising 5 repeats of the GAL4 DNA-binding domain was purified by agarose gel electrophoresis. This DNA fragment was inserted into the blunt-ended plasmid pGCC-LUC, and a clone in which the GAL4 sequence is oriented in the forward direction was selected to construct the reporter gene pGAL4-GCC-LUC (see Fig. 8).

(4) Gene introduction using particle gun

Gold particles (510 μg, diameter 1 mm, Bio-Rad) were coated with 1.6 μg of the reporter gene pGAL4-LUC, 1.2 μg of DNA of the effector plasmid pGAL4DB-SUP or a deletion thereof, pGAL4DB-175/204SUP, 1.2 μg of the control plasmid p35S-AtERF5 or pGAL4DB, and 0.32 μg of the reference gene plasmid. *Arabidopsis thaliana* leaves (7 leaves) on the 21st day of growing were laid out in a 9-cm petri dish on a water-moistened filter paper, and DNA was introduced therein using the PDS 1000/HE

device for particle bombardment (Bio-Rad). Subsequently, the leaves were allowed to stand at 22°C for 6 hours in a well-lit place, and reporter gene activity was then assayed.

(5) Assay of luciferase activity

The *Arabidopsis thaliana* leaves that had been allowed to stand for 6 hours were grinded in liquid nitrogen, allowed to become suspended in 200 μl of the Passive Lysis Buffer from the Dual-Luciferase® Reporter Assay System (Promega), and then centrifuged to recover the supernatant. This cell extract (20 μl) was mixed with 100 μl of the assay buffer attached to the Dual-Luciferase® Reporter Assay System (Promega), and luciferase activity was assayed using a luminometer (TD 20/20, Turner Design). Activity of firefly luciferase and that of *Renilla* luciferase were assayed by measuring luminescence over 10 seconds in the integral mode in accordance with the instructions of the assay kit. A value indicating reference gene activity was divided by a value indicating reporter gene activity, and the relative value thereof, i.e., the relative luciferase activity, was determined as a measured value. Each type of effector plasmid was independently subjected to repeat a transient assay three times, and the mean and the standard deviation were determined. The relative activity value of the reporter gene p35S-GAL4-LUC without the effector plasmid was set to be 100, and the relative activity value of the reporter gene pGAL4-GCC-LUC was set to be 1. Effects of the effector plasmid were then inspected based on variations in values indicating reporter gene activity when the effector plasmid was simultaneously introduced into cells.

Fig. 9A shows the reporter gene and the effector plasmid, wherein 5XGAL4 represents the DNA-binding sequence of the GAL4 transcription factor; TATA represents a region comprising the TATA box of the CaMV 35S promoter; LUC represents a luciferase gene; CaMV 35S represents the 35S protein gene promoter derived from the cauliflower mosaic virus: GAL4DB represents a region encoding DNA-binding domain of an yeast GAL4 transcription factor; and Nos represents the transcription terminator of the nopaline synthase gene.

Fig. 9B shows the effects of SUP and deleted SUP on reporter gene activity (relative activity), wherein numerical values on the left (such as 175/204) indicate the

residues constituting an amino acid sequence of SUP, the box in the center represents an amino acid sequence corresponding to the numerical values on the left, and the graph on the right represents reporter gene activity upon introduction of the effector plasmid having the region shown on the left.

According to the results shown in Fig. 9B, the value indicating reporter gene activity decreases upon co-expression of of the reporter gene p35S-GAL4-LUC and the effector plasmid pGAL4DB-SUP. This indicates that pGAL4DB-SUP has the effect of repressing activity of reporter gene (functions as a repressor). The effector plasmid pGAL4DB-SUP caused the reporter gene activity to decrease by 75%, in comparison with the activity when the effector was not introduced (i.e., the control) (Fig. 9B, 1/204). The control p35S-GAL4DB that did not contain any encoding region of SUP did not suppress the activity of the reporter gene. This indicates that SUP serves as a repressor of transcription.

The effector plasmid pGAL4DB-175/204SUP having a DNA fragment from which the protein-encoding region of the SUP gene had been deleted exhibited greater repressing effects than the case where pGAL4DB-SUP had been expressed. Thus, this effector plasmid was found to repress 95% of reporter gene activity (Fig. 9B, 175/204SUP).

This result demonstrates that the domain having functions as a repressor of SUP (the repression domain) existed in the amino acid sequence (the 175/204 region) of SUP. This amino acid sequence is shown in SEQ ID NO: 61.

Fig. 10A shows the reporter gene pGAL4-GCC-LUC and a variety of effector plasmids.

According to Fig. 10B, reporter gene activity became at least 15 times higher when the reporter gene pGAL4-GCC-LUC and the effector plasmid p35S-AtTERF5 that was known to be capable of activating transcription were expressed into *Arabidopsis thaliana* leaves using a particle gun, in comparison with the case where effector plasmid was not introduced (= 1). When the reporter gene was co-expressed with p35S-AtERF5 and pGAL4DB-SUP, the reporter gene activity was increased as low as approximately

2.5 times. This indicates that the SUP protein has the effect of repressing 84% (1-2.5/15) of the activity of AtERF5 for activating transcription. Further, when pGAL4DB-175/204SUP having a region deleted from SUP was co-expressed with the reporter gene GAL4-GCC-LUC and the effector plasmid pATERF5, 90% of the reporter gene activity was repressed. When pGAL4DB was expressed, elevation of reporter gene activity by p35S-AtERF5 was not affected. This indicates that the effect of the reporter gene for repressing transcription is due to the SUP protein.

(Example 3) Effects of gene encoding the 175/204 repression domain of SUP in repressing transcriptions activated by EIN3 in plants

(1) Construction of transformation vector pBIG2

Plasmid p35S-GFP (Clontech, U.S.A.) was cleaved with restriction enzymes, *Hin*dIII and *Bam*HI, a DNA fragment comprising the cauliflower mosaic virus 35S promoter (CaMV 35S) was separated by agarose gel electrophoresis, and the separated fragment was recovered. The plant transformation vector pBIG-HYG assigned from Michigan State University, U.S.A. (Becker, D., 1990, Nucleic Acid Research, 18: 203) was cleaved with restriction enzymes, *Hin*dIII and *Sst*I, and a DNA fragment from which the GUS gene had been removed by agarose gel electrophoresis was obtained.

The following DNA strands were synthesized, incubated at 70°C for 10 minutes, and annealed by natural cooling to prepare double-stranded DNA. This DNA fragment comprises *Bam*HI restriction site at its 5' terminus, the omega sequence derived from the tobacco mosaic virus for improving translation efficiency, and the *Sma*I and *Sal*I restriction sites.

5'-GATCCACAATTACCAACAACAACAAACAACAAACAACATTACAATTA CAGATCCCGGGGGTACCGTCGACGAGCTC-3' (SEQ ID NO: 41)

5'-CGTCGACGGTACCCCCGGGATCTGTAATTGTAATGTTGTTTGTTGTTT GTTGTTGTTGTTGGTAATTGTG-3' (SEQ ID NO: 42)

A DNA fragment comprising the CaMV 35S promoter region and the synthesized double-stranded DNA were inserted into the *Hin*dIII-*Sst*I site of pBIG-HYG

from which the GUS gene had been removed. Thus, the plant transformation vector pBIG2 was obtained.

(2) Construction of transformation vector pEIN3SUPRD

PCR was carried out using the clone pEIN3 comprising the full-length cDNA of EIN3 assigned from the Salk Institute, U.S.A. as a template and using the 5'-primer 8: AATGATGTTTAATGAGATGGG (SEQ ID NO: 43) and the 3'-primer 9: ATGAATCCCCGGGATATTATTC (SEQ ID NO: 44) to amplify a DNA fragment comprising the nucleotide sequence (the 1/485 region) corresponding to the region encoding the amino acid sequence (the 1/162 region) of EIN3. The amplified fragment was cleaved with a restriction enzyme, *Sma*I, and the DNA fragment of interest was isolated by agarose gel electrophoresis. This fragment was inserted into pBIG2, which had been cleaved with a restriction enzyme, *Sma*I, and a clone that had been cloned in the forward direction was isolated to obtain pBIG2/EIN3-1/162.

The clone pEIN3 comprising the full-length cDNA of EIN3 was cleaved with restriction enzymes, *Sma*I and *Pst*I, and a DNA fragment (the 487/1695 region) encoding the amino acid sequence (the 163/565 region) of EIN3 was isolated by agarose gel electrophoresis. This DNA fragment was inserted into the cloning vector pBluescriptII that had been cleaved with restriction enzymes, *Sma*I and *Pst*I to prepare the plasmid pEIN3-163/565.

PCR was carried out using the clone pEIN3 comprising the full-length cDNA of EIN3 as a template and using the 5'-primer 10: CGACACTGCAGATCACAAC (SEQ ID NO: 45) and the 3'-primer 11: ATCCCGAACCATATGGATACATCTTGCTGC (SEQ ID NO: 46) in which the stop codon TAA at the 3-terminus had been converted to CCC to amplify a DNA fragment comprising the nucleotide sequence (the 1696/1884 region) corresponding to the region encoding the amino acid sequence (the 566/628 region) of EIN3. The amplified fragment was cleaved with a restriction enzyme, *Pst*I, and the fragment was isolated by agarose gel electrophoresis.

In the same manner as in the case of Example 2 (2) (2-2) above, a DNA fragment comprising the nucleotide sequence (the 523/612 region) corresponding to the region

encoding the amino acid sequence (the 175/204 region) of SUP having the restriction *Sal*I site at its 3' terminus and a DNA fragment comprising the nucleotide sequence (the 1696/1884 region) corresponding to the region encoding the amino acid sequence (the 566/628 region) of EIN3, each of which had been designed to have the amino acid reading frame to be in-frame with that of the other, were inserted into the aforementioned pEIN3-163/565 that had been cleaved with restriction enzymes, *Pst*I and *Sal*I to prepare pEIN3-163/628-SupRD.

The plasmid pEIN3-163/628-SupRD was cleaved with restriction enzymes, *Sma*I and *Sal*I, and a DNA fragment encoding the amino acid sequence (the 163/628 region) of EIN3 and the 175/204 region of SUP was isolated by agarose gel electrophoresis. The isolated fragment was inserted into the pBIG2-EIN3-1/162 prepared by cleaving the pBIG2-EIN3-1/162 with a restriction enzyme *Sma*I, and the transformation vector pEIN3SUPRD comprising 35S-EIN3-SupRD-Nos was obtained.

(3) Transformation of plant with pEIN3SUPRD

Arabidopsis thaliana was transformed with pEIN3SUPRD in accordance with the protocol "Transformation of *Arabidopsis thaliana* by vacuum infiltration (http://www.bch.msu.edu/pamgreen/protocol.htm)," except that infiltration was carried out only by immersing *Arabidopsis* in infiltration medium without putting it under vacuum.. The plasmid pEIN3RD was introduced into soil bacteria (the *Agrobacterium tumefaciens* strain GV3101 (C58C1Rifr) pMP90 (Gmr) (koncz and Schell 1986)) by an electroporation. The introduced cells were cultured in a 1-liter YEP medium (Table 2 below) until the OD600 became to be 1.

(Table 2)

YEP medium (1 liter)	
10 g	Bactopeptone
10 g	Yeast extract
5 g	NaCl

Subsequently, cells were colleted from the culture solution and then suspended in 1 liter of infiltration medium (Table 3 below).

(Table 3)

Infiltration medium (1 liter)	
2.29 g	MS salt
50 g	Sucrose
0.5 g	MES to pH 5.7 with KOH
0.044 μM	Benzylaminopurine
0.2 ml	Silwet L-77

Arabidopsis thaliana that had been grown for 14 days was immersed in this solution for 1 minute for infiltration, and the infiltrated *Arabidopsis thaliana* was then allowed to grow until fruition. The colleted seeds were sterilized with a 50% bleach/0.02% Triton X-100 solution for 7 minutes, rinsed three times with sterilized water, and then sowed on the sterilized selection medium containing hygromycin (Table 4 below).

(Table 4)

Hygromycin selection medium	
4.3 g/l	MS salt
1%	Sucrose
0.5 g/l	MES to pH 5.7 with KOH
0.8%	Phytagar
30 g/ml	Hygromycin
500 ml	Vancomycin

Transgenic plants to be grown on the aforementioned hygromycin plate were selected and then planted in soil to obtain the next-generation seeds.

(4) Inspection of ethylene sensitivity of transgenic plant

The seed of the second generation of the transgenic plants (T2) were sowed on the sterilized growth medium-containing MS plate (Table 5 below) comprising an ethylene precursor 1-aminocyclopropane-D-carboxylic acid (ACC, final concentration of 10 μM).

(Table 5)

Selection medium-containing MS plate	
4.3 g/l	MS salt
1%	Sucrose
0.5 g/l	MES to pH 5.7 with KOH
0.8%	Phytagar ACC (final 10 μM)

The aforementioned seeds were incubated at 4°C for 3 days and then allowed to grow in a dark place at 22°C for 3 days. In accordance with a conventional technique, the triple response, i.e., an ethylene-responsive phenotype, was observed in etiolated seedlings. The results are shown in Fig. 11 and Fig. 12.

According to Fig. 11 and Fig. 12, wild-type Col-0 strains exhibited the triple response such as the curvature of the apical hook and an inhibition of root elongation in the presence of ACC. The plants transformed with pEIN3SUPRD (Fig. 11; 35S::EIN3SUPRD), however, exhibited no ethylene-responsive phenotype, i.e., the curvature of the apical hook or an inhibition of root elongation did not occur, as with the case of the ein3 mutant that were EIN3 mutants (Fig. 11; ein3-1). Wild-type strains that had been grown under ordinary light became dwarf plants in the constant presence of ethylene gas (100 ppm, 1 ml/min) (i.e., an ethylene-responsive phenotype). In contrast, plants transformed with pEIN3SUPRD (Fig. 12; 35S::EIN3SUPRD) became somewhat larger than the ein3 mutants (Fig. 12; ein3-1).

Expression of the PDF1.2 gene, that of the basic chitinase (BCHN) gene, and that of the ethylene responsive factor 1 (ERF1) gene, of which expression are induced by ethylene in the case of the wild-type strain but are not induced in the case of the ein3 strain, i.e., an ethylene-insensitive mutant, were investigated by Northern blot hybridization of RNAs isolated from the wild-type strains and from the plants transformed with pEIN3SUPRD.

As a result, expression of the PDF1.2, that of ERF1, and that of BCHN genes were induced in the wild-type strain (Fig. 13; Col-0) when treated with ethylene (100 ppm ethylene gas for 12 hours) as shown in Fig. 13. As in the case of the ein3 mutant, expression of the PDF1.2, that of ERF1, and that of BCHN genes were not induced in the plant transformed with pEIN3SUPRD (Fig. 13; 35S::EIN3SUPRD) even when they were treated with ethylene. Such transgenic plants exhibited ethylene-insensitive phenotype (in the Figure, "*EF*" (elongation factor) indicates the endogenous control).

As is apparent from the foregoing, a peptide having the amino acid sequence (the 175/204 region) of SUP and a gene encoding the same were capable of converting

any transcription factor into a transcriptional repressor.

(Example 4) Effects of gene encoding the 191/225 repression domain of ERF3 on the repression of the transcription activated by EIN3 in plants

(1) Construction of transformation vector pBIG2

The transformation vector pBIG2 was constructed in the same manner as in Example 3 (1).

(2) Construction of transformation vector pEIN3RD

PCR was carried out using the clone pEIN3 comprising the full-length cDNA of EIN3 assigned from the Salk Institute, U.S.A. as a template and using the 5'-primer 8: AATGATGTTTAATGAGATGGG (SEQ ID NO: 43) and the 3'-primer 9: ATGAATCCCCGGGATATTATTC (SEQ ID NO: 44) to amplify a DNA fragment comprising the nucleotide sequence (the 1/485 region) corresponding to the region encoding the amino acid sequence (the 1/162 region) of EIN3. The amplified fragment was cleaved with a restriction enzyme, *Sma*I, and the DNA fragment of interest was isolated by agarose gel electrophoresis. This fragment was inserted into pBIG2 that had been cleaved with a restriction enzyme, *Sma*I, and a clone that had been cloned in the forward direction was isolated to obtain pBIG2/EIN3-1/162.

The clone pEIN3 comprising the full-length cDNA of EIN3 was cleaved with restriction enzymes, *Sma*I and *Pst*I, and the DNA fragment (the 487/1695 region) encoding the amino acid sequence (the 163/565 region) of EIN3 was isolated by agarose gel electrophoresis. This DNA fragment was inserted into the cloning vector pBluescriptII that had been cleaved with restriction enzymes, *Sma*I and *Pst*I to prepare the plasmid pEIN3-163/565.

PCR was carried out using the clone pEIN3 comprising the full-length cDNA of EIN3 as a template and using the 5'-primer 10: CGACACTGCAGATCACAAC (SEQ ID NO: 45) and the 3'-primer 11: ATCCCGAACCATATGGATACATCTTGCTGC (SEQ ID NO: 46) in which the stop codon TAA at the 3-terminus had been converted to CCC to amplify a DNA fragment comprising the nucleotide sequence (the 1696/1884 region)

corresponding to the region encoding the amino acid sequence (the 566/628 region) of EIN3. The amplified fragment was cleaved with a restriction enzyme, *Pst*I and then isolated by agarose gel electrophoresis.

A DNA fragment of the nucleotide sequence (the 571/675 region) corresponding to the region encoding the amino acid sequence (the 191/225 region) of ERF3 having the restriction enzyme, *Sal*I site at its 3' terminus was designed to have a reading frame to be in-frame with the carboxyl terminus of EIN3. The nucleotide sequence of the full-length ERF3 gene and the amino acid sequence thereof are shown in SEQ ID NO: 53.

PCR was carried out using the 5'-primer 12: AGTGGGTCCTACTGTGTCGGACTC (SEQ ID NO: 47; bound to the nucleotide sequence (the 569/593 region) of ERF3) and the 3'-primer 13: CCAAATAACATTATCGGTCGACTCAAAATTCCATAGGTG (SEQ ID NO: 48; bound to the nucleotide sequence (the 661/678 region) of ERF3) having the restriction enzyme, *Sal*I site to obtain a DNA fragment comprising the nucleotide sequence (the 571/675 region) corresponding to the region encoding the amino acid sequence (the 191/225 region) of ERF3. This DNA fragment was digested with a restriction enzyme, *Sal*I, and the DNA fragment of interest was isolated by agarose gel electrophoresis.

A DNA fragment encoding the repression domain of ERF3 and a DNA fragment of the nucleotide sequence (the 1696/1884 region) encoding the amino acid sequence (the 566/628 region) of EIN3 were inserted into the aforementioned pEIN3-163/565 that had been cleaved with restriction enzymes, *Pst*I and *Sal*I to prepare pEIN3-163/628-RD.

The plasmid pEIN3-163/628-RD was cleaved with restriction enzymes, *Sma*I and *Sal*I, and a DNA fragment encoding the amino acid sequence (the 163/628 region) of EIN3 and the 191/225 region of ERF3 was isolated by agarose gel electrophoresis. The isolated fragment was inserted into the pBIG2-EIN3-1/162 prepared by cleaving the pBIG2-EIN3-1/162 with a restriction enzyme, *Sma*I, and the transformation vector pEIN3RD comprising 35S-EIN3-RD-Nos was obtained.

(3) Construction of a control transformation vector

In the same manner as described above, a DNA fragment encoding RDm having a mutated domain in which aspartic acids at 215 and 217 in the 191/225 repression domain of ERF3 of the sequence shown below had been substituted with alanines was inserted to obtain pEIN3RDm.

VGPTVSDSSSAVEENQYDGKRDIALALNLAPPMEF (SEQ ID NO: 49)

The following DNA strands encoding the mutated domain including an alanine substitution were synthesized.

5'-AGTGGGTCCTACTGTGTCGGACTCGTCCTCTGCAGTGGAAGAGAACC AATATGATGGGGAAAAGAGGAATTGATCTTGATCTTAACCTTGCTCCACCTATG GAATTTTGAG-3' (SEQ ID NO: 50)

5'-TCGACTCAAAATTCCATAGGTGGAGCAAGGTTAAGATCAAGATCAAT TCCTCTTTTCCCCCATCATATTGGTTCTCTTCCACTGCAGAGGACGAGTCCGACA CAGTAGGACCCACT-3' (SEQ ID NO: 51)

(4) Transformation of plants with pEIN3RD

In the same manner as in Example 3 (3), *Arabidopsis thaliana* was transformed using the transformation vector pEIN3PRD and the control transformation vector pEIN3RDm, and selection was carried out using the hygromycin plate to obtain the next-generation seeds.

(5) Investigation of ethylene sensitivity of transgenic plant

The next-generation seeds of the transgenic plants (T2) were allowed to grow in the same manner as in Example 3 (4) to observe the triple response, i.e., an ethylene-responsive phenotype, in the etiolated seedlings. Under the same conditions, ethylene responsiveness of wild-type Col-0 strains and that of the ein3 mutants were analyzed. The results are shown in Fig. 11 and Fig. 12.

According to Fig. 11, wild-type Col-0 strains exhibited the triple response such as the curvature of the apical hook and an inhibition of root elongation in the presence of ACC. The plants transformed with pEIN3-RDm (Fig. 11; 35S::EIN3RDm) having RDm with a mutated domain in which aspartic acids at 215 and 217 in the 191/225 repression domain of ERF3 had been substituted with alanines exhibited ethylene

responsiveness similar to that of the wild-type Col-0 strains. The plants transformed with pEIN3RD (Fig. 11; 35S::EIN3RD), however, exhibited ethylene insensitive phenotype. That is, neither the curvature of the apical hook or an inhibition of root elongation occurred, as with the case of the ein3 mutant (Fig. 11; ein3-1).

According to Fig. 12, wild-type strains that had been grown under ordinary light became dwarf plants (Fig. 12; Col-1) in the constant presence of ethylene gas (100 ppm for 12 hours) (i.e., an ethylene-responsive phenotype). In contrast, plants transformed with pEIN3RD (Fig. 12; 35S::EIN3RD) became substantially the same size as the ein3 mutant (Fig. 12; ein3-1).

Expression of the PDF1.2 gene, that of the basic chitinase (BCHN) gene, and that of the ethylene responsive factor 1 (ERF1) gene, which are induced by ethylene in the case of the wild-type strain but are not induced in the case of the ein3 mutant, i.e., an ethylene-insensitive mutant, were investigated by Northern blot hybridization of RNAs isolated from the wild-type strains and from the plants transformed with pEIN3RD.

As a result, expression of the PDF1.2, that of ERF1, and that of BCHN genes were induced in the wild-type strains treated with ethylene (100 ppm ethylene gas for 12 hours) as shown in Fig. 13. As with the case of the ein3 mutant, expression of the PDF1.2, that of ERF1, and that of BCHN genes were not induced in the plant transformed with pEIN3RD even when they were treated with ethylene. Such transgenic plants exhibited ethylene-insensitive phenotype.

As is apparent from the foregoing, a peptide having the amino acid sequence (the 191/225 region) of ERF3 and a gene encoding the same were capable of converting any transcription activator into a transcriptional repressor.

(Example 5) Effects of gene encoding the peptide DLDLELRLGFA (corresponding to the 194/204 repression domain of SUP (SRD)) in repressing functions of CUC1 to activate transcription in plants

(1) Construction of transformation vector pBIG2

The transformation vector pBIG2 was constructed in the same manner as in

Example 3 (1).

(2) Construction of transformation vector pCUC1SRD

The following complementary strands (3' complement DNA) were synthesized to prepare the amino acid sequence (VSVWPFTLDLDLELRLGFA). In this amino acid sequence, the amino acid peptide DLDLELRLGFA (referred to as "SRD") was bound to the carboxyl terminus of the protein-encoding region (SEQ ID NO: 54) of the cup-shaped cotyledon 1 (CUC1) transcription factor. Also, the reading frame of the sequence in which the stop codon had been deleted from the coding region of the CUC1 gene was designed to be in-frame with the reading frame of the coding region of SRD.

5'-TTAAGCGAAACCCAAACGGAGTTCTAGATCCAGATCGAGAGTAAAG GGCCACACACTCAC-3' (SEQ ID NO: 55)

Separately, the following DNA sequence corresponding to the 5' region in the protein-encoding region of the CUC1 gene was synthesized.

5'-GGGATGGATGTTGATGTGTTTAACGG-3' (SEQ ID NO: 56)

PCR was carried out using these two single-stranded DNAs as a primer and using the clone comprising the full-length cDNA of CUC1 assigned from Professor Tasaka of the Nara Institute of Science and Technology as a template to prepare the CUC1SRD gene in which the CUC1 coding region is fused with SRD. PCR conditions are as described above.

The DNA fragment of interest was isolated from the obtained DNA sample by agarose gel electrophoresis, the isolated fragment was inserted into pBIG2 that had been cleaved with a restriction enzyme, *Sma*I, and a clone that had been cloned in the forward direction was isolated to obtain p35S::CUC1SRD.

(3) Preparation of plants transformed with p35S::CUC1SRD

Arabidopsis thaliana was transformed with p35S::CUC1SRD in the same manner as in Example 3 (3).

(4) Phenotype of the germinated transgenic plants

Phenotype of the resulting germinated transgenic plants (35S::CUC1SRD) are shown in Fig. 15 (right).

As a control, the cotyledon traits of the wild-type Col-0 strain and those of the cuc1/cuc2 double mutant are shown in Fig. 15 (left) and Fig. 15 (middle), respectively.

The cotyledon of the wild-type Col-0 strain was divided into two pieces, and fusion was not observed in either the base or leaf body. In the case of the cuc1/cuc2 double mutant, however, two pieces of cotyledons were fused with each other at substantially all regions at both ends, and a cup-like shape was exhibited (a cup-shaped cotyledon).

Some or most regions of the cotyledons were fused in all the germinated *Arabidopsis thaliana* transformed with p35S::CUC1SRD that were grown in the presence of hygromycin. This feature is very similar to that of a cuc1/cuc2 double mutant. A phenotype of partial fusion of cotyledons was substantially the same as that of the cuc1/stm-1 double mutant (Plant Cell, 9, 841, 1997; Development, 126, 1563, 1999; Development, 128, 1127, 2000). Further, formation of the apical meristem was not observed in most of these transgenic plants.

Accordingly, a peptide having the amino acid sequence DLDLELRLGFA (corresponding to the 194/204 repression domain of SUP) and a gene encoding the same peptide were found to be capable of converting any transcription factor into a transcriptional repressor.

(Example 6) Effects of gene encoding the peptide LDLELRLGFA (corresponding to the 195/204 repression domain of SUP (SRD1)) and the peptide LDLNLAPPMEF (corresponding to the 215/225 repression domain of ERF3 (RD1)) in repressing of the transcription activated by EIN3 in plants

(1) Construction of transformation vector pBIG2

The transformation vector pBIG2 was constructed in the same manner as in Example 3 (1).

(2) Construction of transformation vector pEIN3SUPRD

PCR was carried out using the clone pEIN3 comprising the full-length cDNA of EIN3 assigned from the Salk Institute, U.S.A. as a template and using the 5'-primer 8:

AATGATGTTTAATGAGATGGG (SEQ ID NO: 43) and the 3'-primer 9: ATGAATCCCCGGGATATTATTC (SEQ ID NO: 44) to amplify a DNA fragment comprising the nucleotide sequence (the 1/485 region) corresponding to the region encoding the amino acid sequence (the 1/162 region) of EIN3. The amplified fragment was cleaved with a restriction enzyme, *Sma*I, and the DNA fragment of interest was isolated by agarose gel electrophoresis. This fragment was inserted into pBIG2 that had been cleaved with a restriction enzyme, *Sma*I, and a clone that had been cloned in the forward direction was isolated to obtain pBIGII/EIN3-1/162.

The clone pEIN3 comprising the full-length cDNA of EIN3 was cleaved with restriction enzymes *Sma*I and *Pst*I, and the DNA fragment (the 487/1695 region) encoding the amino acid sequence (the 163/565 region) of EIN3 was isolated by agarose gel electrophoresis. This DNA fragment was inserted into the cloning vector pBluescriptII that had been cleaved with restriction enzymes, *Sma*I and *Pst*I to prepare the plasmid pEIN3-163/565.

PCR was carried out using the clone pEIN3 comprising the full-length cDNA of EIN3 as a template and using the 5'-primer 10: CGACACTGCAGATCACAAC (SEQ ID NO: 45) and the 3'-primer 11: ATCCCGAACCATATGGATACATCTTGCTGC (SEQ ID NO: 46) in which the stop codon TAA at the 3-terminus had been converted to CCC to amplify a DNA fragment comprising the nucleotide sequence (the 1696/1884 region) corresponding to the region encoding the amino acid sequence (the 566/628 region) of EIN3. The amplified fragment was cleaved with a restriction enzyme, *Pst*I and then isolated by agarose gel electrophoresis.

Two DNA strands, each of which had been designed to encode the amino acid sequence of SRD1 (LDLELRLGFA) having the restriction site of *Sal*I at the 3' terminus and to have the amino acid reading frame to be in-frame with that of GAl 4DBD, were prepared in the same manner as in Example 2 (2) (2-2).

5'-CCTGGATCTAGAACTCCGTTTGGGTTTCGCTTAA-3' (SEQ ID NO: 57)

5'-TCGACTTAAGCGAAACCCAAACGGAGTTCTAGATCCAGG-3' (SEQ ID NO: 58)

The above two DNA strands were annealed in the manner described in Example 2 to obtain two double-stranded DNA.

Separately, two another DNA strands, each of which had been designed to encode the amino acid sequence of RD1 (LDLNLAPPMEF) having the restriction site of *Sal*I at the 3' terminus and to have the amino acid reading frame to be in-frame with that of GAl 4DBD, were prepared.

5'-CCTTGATCTTAACCTTGCTCCACCTATGGAATTTTGA-3' (SEQ ID NO: 59)

5'-TCGACTCAAAATTCCATAGGTGGAGCAAGGTTAAGATCAAGG-3' (SEQ ID NO: 60)

The above another two DNA strands were annealed in the similar manner to obtain two double-stranded DNA. Each of the obtained DNAs and a DNA fragment comprising the nucleotide sequence (the 1696/1884 region) corresponding to the region encoding the amino acid sequence of EIN3 were inserted into the aforementioned pEIN3-163/565 which had been prepared by cleaving with restriction enzymes, *Pst*I and *Sal*I to construct plasmids pEIN3-163/628-SRD1 and pEIN3-163/628-RD1. The plasmids pEIN3-163/628-SRD1 and pEIN3-163/628-RD1 were cleaved with restriction enzymes, *Sma*I and *Sal*I, and a DNA fragment in which a region encoding SRD1 or RD1 has been fused with the amino acid sequence (the 163/628 region) of EIN3 was isolated by agarose gel electrophoresis. The isolated fragment and pBIG2-EIN3-1/162 obtained in the same manner as in Example 3 (2) were inserted into pBIG2-EIN3-1/162, which had been cleaved with a restriction enzyme, *Sma*I to obtain transformation vectors pEIN3SRD1 and pEIN3RD1 independently comprising CaMV35S-EIN3-SupRD-Nos.

(3) Preparation of plants transformed with pEIN3SRD1 or pEIN3RD1

Arabidopsis thaliana was transformed with pEIN3SRD1 or pEIN3RD1 in the same manner as in Example 3 (3) to obtain the next-generation seeds.

(4) Investigation of ethylene sensitivity of transgenic plants

The triple response of etiolated seedlings was investigated in the same manner as in Example 3 (4). The results are shown in Fig. 16 and Fig. 17.

According to Fig. 16 and Fig. 17, wild-type Col-0 strains exhibited the triple response such as the curvature of the apical hook and an inhibition of root elongation in the presence of ACC. The plants transformed with pEIN3SRD1 or pEIN3RD1 (Fig. 16; 35S::EIN3SRD1 or 35S::EIN3RD1), however, exhibited ethylene-insensitive phenotype. That is, neither the curvature of the apical hook or an inhibition of root elongation occurred, as with the case of the ein3 mutant (Fig. 16; ein3-1). Wild-type strains that had been grown under ordinary light became dwarf plants in the constant presence of ethylene gas (100 ppm) (i.e., an ethylene-responsive physiological phenomenon). In contrast, plants transformed with pEIN3SRD1 or pEIN3RD1 (Fig. 17; SRD1 or RD1) became the same size as the plant obtained by growing the ein3 mutant that showed ethylene-insensitive phenotype (Fig. 17; ein3) in the presence of ethylene.

As is apparent from the above results, a peptide having the amino acid sequences LDLELRLGFA and LDLNLAPPMEF and a gene encoding the same peptide were capable of converting any transcription factor into a transcriptional repressor.

(Example 7) Functional conversion of the transcription factor production-of-anthocyanin-pigment 1 (PAP1) in plants caused by the gene encoding a peptide LDLDLELRLGFA (corresponding to the 193/204 repression domain of SUP (SRDX))

(1) Construction of transformation vector p35S::PAP1SRDX

(1-1) Isolation of PAP1 cDNA

A DNA fragment comprising the PAP1 coding region but not containing the stop codon was obtained from the *Arabidopsis thaliana* cDNA library. This fragment was amplified by PCR using the following primers. The amplified fragment was separated by agarose gel electrophoresis and then recovered. PCR conditions are as described in Example 3.

5' Primer: 5'-AAAATGGAGGGTTCGTCCAAAGGGCTGCGAAAAGG-3' (SEQ ID NO: 62)

3' Primer: 5'-ATCAAATTTCACAGTCTCTCCATCGAAAAGACTC-3' (SEQ ID

NO: 63)

The obtained cDNA of the PAP1 gene and the amino acid sequence encoded thereby are shown in SEQ ID NO: 66 in the Sequence Listing.

(1-2) Synthesis of gene encoding peptide LDLDLELRLGFA (SRDX)

The following DNA strands that were designed to encode a 12-amino acid peptide LDLDLELRLGFA (SRDX) and to have the stop codon TAA at its 3' terminus were synthesized. The synthesized DNAs were annealed in the same manner as in Example 3 to prepare double-stranded DNA.

5'-CTGGATCTGGATCTAGAACTCCGTTTGGGTTTCGCTTAAG-3' (SEQ ID NO: 64)

5'-CTTAAGCGAAACCCAAACGGAGTTCTAGATCCAGATCCAG-3' (SEQ ID NO: 65)

(1-3) Preparation of transformation vector

The DNA fragment consisting of the protein-encoding region of the PAP1 gene obtained above and a DNA fragment comprising the coding region of SRDX were inserted into pBIG2 that had been cleaved with a restriction enzyme, *Sma*I in the same manner as in Example 3. A clone that had been cloned in the forward direction was isolated to obtain the transformation vector p35S::PAP1SRDX.

(2) Preparation of plants transformed with transformation vector p35S::PAP1SRDX

The next-generation seeds of *Arabidopsis thaliana* transformed with the aid of the transformation vector p35S::PAP1SRDX and the wild-type (Col-0) seeds as the control were sowed on the MS agar medium containing 3% sucrose and the MS agar medium containing no sucrose, and these seeds were allowed to grow under the same conditions as in Example 3. As a result, the wild-type seedlings accumulated reddish-purple pigments (a feature of anthocyanin) in a MS agar medium containing 3% sucrose which applies stress to the strains. In contrast, the *Arabidopsis thaliana* seedlings transformed with p35S::PAP1SRDX did not accumulate such pigments (Fig. 18A).

Expression of the dihydroflavonol reductase (DFR) gene, that is involved in

phenylpropanoid synthesis, i.e., an anthocyanin biosynthesis, was investigated by RT-PCR described in the Reference Example below. As a result, expression of this gene was enhanced simultaneously under stress condition in the case of wild-type strains. In contrast, such enhanced expression was not observed in the *Arabidopsis thaliana* transformed with p35S::PAP1SRDX (Fig. 18). This indicates that the PAP1 transcription factor to which the peptide SRDX had been applied was converted into a repressor, and the resulting the repressor suppresses the expression of the DFR gene in plants to inhibit anthocyanin synthesis. This also indicates that such system for repressing gene expression utilizing a repressor is applicable to the inhibition of synthesis of secondary metabolites.

(Example 8) Functional conversion of AtMYB23 transcription factor in plants by gene encoding peptide LDLDLELRLGFA (SRDX)

(1) Construction of transformation vector p35S::AtMYB23SRDX

(1-1) Isolation of AtMYB23 cDNA

A DNA fragment comprising the AtMYB23 coding region but not comprising the stop codon was obtained from the *Arabidopsis thaliana* cDNA library. This fragment was amplified by PCR using the following primers. The amplified fragment was separated by agarose gel electrophoresis and then recovered. PCR conditions are as described in Example 3.

5' Primer: 5'-AAAATGAGAATGACAAGAGATGGAAAAGAACATG-3' (SEQ ID NO: 67)

3' Primer: 5'-AAGGCAATACCCATTAGTAAAATCCATCATAGTG -3' (SEQ ID NO: 68)

The obtained cDNA of the AtMYB23 gene and the amino acid sequence encoded thereby are shown in SEQ ID NO: 69 in the Sequence Listing.

(1-2) Synthesis of gene encoding peptide LDLDLELRLGFA (SRDX)

The following DNA strands that were designed to encode a 12-amino acid peptide LDLDLELRLGFA (SRDX) and to have the stop codon TAA at its 3' terminus

were synthesized. The synthesized strands were annealed in the same manner as in Example 3 to prepare double-stranded DNA.

5'-CTGGATCTGGATCTAGAACTCCGTTTGGGTTTCGCTTAAG-3' (SEQ ID NO: 64)

5'-CTTAAGCGAAACCCAAACGGAGTTCTAGATCCAGATCCAG-3' (SEQ ID NO: 65)

(1-3) Preparation of transformation vector

The DNA fragment consisting of the protein-encoding region of the AtMYB23 gene obtained above and a DNA fragment comprising the SRDX-encoding region were inserted into pBIG2 that had been cleaved with a restriction enzyme, *Sma*I in the same manner as in Example 3. A clone that had been cloned in the forward direction was isolated to obtain the transformation vector p35S:: AtMYB23SRDX.

(2) Preparation of plants transformed with transformation vector p35S:: AtMYB23SRDX

The next-generation seeds of *Arabidopsis thaliana* transformed with the aid of the transformation vector p35S:: AtMYB23SRDX and the wild-type (Col-0) seeds as a control were sowed on the MS agar medium, and these seeds were allowed to grow under the same conditions as in Example 3. As a result, plants transformed with p35S:: AtMYB23SRDX were found to have no trichome that was present on the epidermis of a wild-type Col-0 strain. Otherwise, the number of trichomes significantly decreased in comparison with that of the wild-type strain (Fig. 19A).

Also, expression of GLABRA1 (GL1) genes, that of GLABRA2 (GL2) genes, and that of TRANSPARENT TESTA GLABRA 1 (TTG1) genes, which were involved in the formation of trichomes, were investigated by RT-PCR described in the Reference Example below. These three types of genes were expressed similarly in the wild-type strains whereas expression of GL2 was significantly repressed in plants transformed with p35S::AtMYB23SRDX having no trichome (Fig. 19B). This indicates that the AtMYB23 transcription factor comprising a peptide SRDX added thereto is converted into a repressor, and the resultant represses the transcription of the GL2 gene in plants to suppress the formation of trichomes.

(Example 9) Transcriptional repression test in tobacco leaf and petunia

The reporter gene CaMV35S-GAL4::LUC and the effector plasmid CaMV35S::GAL4DBD:RD (the 175/204 repression domain of SUP) were introduced using a particle gun into 1.5 cm-leaves that had been sampled in the same manner as in the case of Example 2 from the plants two weeks after the inoculation of tobacco (*Nicotiana tabacum* BY4) seeds, and allowed to stand on a water-moistened filter paper at 25°C for 16 hours. Luciferase activity, i.e., reporter gene activity, was then assayed. As a result, the reporter gene activity was found to be inhibited by 84% when 35S-GAL4DB-RD was introduced, in comparison with the case of the control where 35S-GAL4DB and the reporter plasmid had been introduced (relative value of 100). Similarly, the aforementioned effector plasmid and the reporter plasmid were introduced using a particle gun into 1.0 cm-leaves sampled from the plants 3 weeks after the inoculation of petunia seeds, and luciferase activity was then assayed. As a result, the reporter gene activity was found to be inhibited by 82% when 35S-GAL4DB-RD was introduced, in comparison with the case of the control where 35S-GAL4DB and the reporter plasmid had been introduced (relative value of 100).

As is apparent from the above results, a chimeric gene having a repression domain was capable of repressing gene transcription in tobacco leaves and in petunia as well as in *Arabidopsis thaliana*.

(Reference Example) Method of analyzing expression of gene by RT-PCR

A method of analyzing expression of gene by RT-PCR employed in Examples 7 and 8 above is described below.

Total RNA was extracted from the *Arabidopsis thaliana* leaves and purified in the manner described above. Total RNA (1.65 μg or 2.5 μg) was prepared therefrom, and subjected to the DNase treatment under the following conditions in order to remove DNA that was also present.

Total RNA	1.65 μg or 2.5 μg

10x DNase Buffer	5 μl
DNase I	1 μl (5 u)
RNase inhibitor	0.5 μl (10 u)
DEPC-treated water	Balance (to bring the total amount to 50 μl)

Reaction was allowed to incubate at 37°C for 30 minutes, a phenol/chloroform solution was added thereto to deactivate each enzyme, and the total RNA from which DNA had been removed via ethanol precipitation was prepared.

Subsequently, the first strand of cDNA was synthesized from the total RNA using the T-Primed First-Strand Kit (Amersham). The total RNA (1.65 μg or 2.5 μg) was allowed to become suspended in 33 μl of DEPC-treated water, the suspension was incubated at 65°C for 5 minutes, and the RNA solution was added to a tube containing the First-Strand Reaction Mix, followed by reaction at 37°C for 60 minutes. Theoretically, the total RNA is considered to have been converted into the first strand cDNA via this reaction.

PCR was carried out using the synthesized first strand cDNA as a template and using a primer specific to the gene to be inspected. The nucleotide sequences of the primers and the composition of the PCR reaction solution used are shown below.

(Nucleotide sequences of primers used in PCR)

β-Tubulin (TUB)

5' Primer: 5'-CGTGGATCACAGCAATACAGAGCC (SEQ ID NO: 70)

3' Primer: 5'-CCTCCTGCACTTCCACTTCGTCTTC (SEQ ID NO: 71)

DFR

5' Primer: 5'-AAAAAGATGACAGGATGGGT (SEQ ID NO: 72)

3' Primer: 5'-CCCCTGTTTCTGTCTTGTTA (SEQ ID NO: 73)

TTG1

5' Primer: 5'-GGGATGGATAATTCAGCTCCAGATTC (SEQ ID NO: 74)

3' Primer: 5'-AACTCTAAGGAGCTGCATTTTG (SEQ ID NO: 75)

GL1

5' Primer: 5'-GGGATGAGAATAAGGAGAAGAGATGAAAAAGAG (SEQ ID NO: 76)

3' Primer: 5'-AAGGCAGTACTCAATATCACTAGAAGCAAAATT (SEQ ID NO: 77)

GL2

5' Primer: 5'-ATGGCCGTCGACATGTCTTCCAAACAACCCACC (SEQ ID NO: 78)

3' Primer: 5'-GCAGGGAGTTCTCGTGCCGTTCTTGAATAG (SEQ ID NO: 79)

(Composition of a PCR reaction solution)

Template cDNA	1 μl (50 ng or 75 ng as total RNA)
10×PCR Buffer	5 μl
2.5 mM dNTP mix	4 μl
5' Primer	0.5 μl (100 pmol/μl)
3' Primer	0.5 μl (100 pmol/μl)
rTaq Polymerase	0.25 μl (1.25 u)
DEPC-treated water	38.75 μl

PCR conditions were varied from the aforementioned conditions as follows: denaturation at 95°C for 2 minutes, 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute. The number of times for repeating this cycle was changed from 25 to 35 depending on the type of gene to be inspected.

Subsequently, the RT-PCR product obtained via the aforementioned procedures was analyzed by Southern blotting and then evaluated semiquantitatively. DNA fragments in amounts of 1/100 to 1/1000 of the DNA amplified by PCR was subjected to agarose gel electrophoresis and then transferred to a nylon membrane. DNA of the corresponding gene was prepared as a probe, labeled using the ECL direct nucleic acid labeling and detection system kit (Amersham), and then subjected to hybridization and

detection. The detected band represents the amount of DNA corresponding to mRNA of the gene to be inspected. Accordingly, the detected band was compared with that of the wile-type and with that of the transformed sample to inspect the expression level of each gene. In this case, the expression level of the β-tubulin (TUB) of the endogenous gene was simultaneously detected as a control.

All publications, patents, and patent applications cited herein are incorporated herein by reference in their entirety.

Industrial Applicability

The peptide of the present invention that is capable of converting a transcription factor into a transcriptional repressor is very short. Thus, it can be very easily synthesized, and it can effectively repress transcription of genes.

When the gene of the present invention is fused with a gene encoding a DNA-binding domain of a transcription factor that binds to a specific target gene, the gene of the present invention can selectively repress the transcription of a specific gene. Such repression appears as a dominant trait, and the gene of the present invention can also repress functions of other transcription factors that are also involved in the transcription of interest. Accordingly, such gene is very useful for analyzing transcriptional functions that have not been elucidated by conventional single-gene knockout technology. It is also applicable to plants having the amphidiploid genome, such as wheat.

When the gene of the present invention is fused with, for example, DNA that binds specifically to the region for regulating the transcription of cancerous genes and is allowed to express in a cell, the gene of the present invention can effectively repress the expression of cancerous genes.

In the case of plants, for example, the gene of the present invention can create a flower having petals of different colors that could not be attained in the past. This can be realized by regulating the expression of genes encoding the pigment-metabolic

enzymes. Also, the gene of the present invention enables the production of low-allergen foods by repressing the expression of allergenic proteins. Furthermore, repression of the expression of genes capable of synthesizing lignin enables the production of trees with low lignin content, thereby producing high-quality pulps. Accordingly, the present invention is applicable to and is able to provide useful technical means for a wide variety of fields.